'

# COLD SPRING HARBOR SYMPOSIA
# ON QUANTITATIVE BIOLOGY

# VOLUME XI

## LIST OF PREVIOUS VOLUMES

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# ON QUANTITATIVE BIOLOGY

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# Heredity and Variation
# in Microorganisms

# FOREWORD

After an interval of three years imposed on us by the war emergency, we are again able to continue with the yearly Symposia on Quantitative Biology. The topic for this meeting was selected two years ago, when it was thought that a symposium could be held in the summer of 1945; the meeting had to be postponed, however, because of travel restrictions. During the last two years, research on problems connected with the genetics and physiology of microorganisms has made very remarkable progress in the laboratories of the United States. Moreover, when contact with continental Europe was re-established we found that during the war many new discoveries in the same field had been made in France. For these reasons, the continued delay of one year was fortunate, because it gave us an opportunity to broaden considerably the scope of our program. When research activity is at a high level, new and important developments occur continuously; and this Symposium was effective as a means of exchange of the most recent information. Acting as a catalyzer, it brought together scientists from American laboratories as well as from the laboratories of Canada, France, England, Denmark, Sweden, and Chile.

This year the program of the meetings was arranged in a logical sequence. Papers dealing with viruses were presented first (Hershey, Anderson, Pirie, and Delbrück and Bailey). These were followed by papers dealing with bacteria (van Neil, Dubos, Bunting, McCarty *et al.*, Lwoff, Luria, Demerec and Latarjet, and Tatum), and with fungi and other microorganisms (Johnson, Pontecorvo, Bonner, Ryan, Spiegelman, Lindegren and Lindegren, Hollaender, and Sonneborn). Finally, the papers of Drs. Kidd, MacDowell, Rhoades, and Richards were given. In this volume, the papers are arranged alphabetically according to authors, for greater ease of reference.

On several occasions during the early sessions of the Symposium, suggestions were made concerning the existence of sexual fusion in bacteria; and Dr. Dubos mentioned in this connection the current work of Dr. Louis Dienes, which indicates that "large bodies" are formed in the region where certain strains of bacteria, plated separately on one plate, meet. We were very fortunate in that Dr. Dienes was able, upon special invitation, to come to our meeting and give us a first-hand report of his discovery. His paper is published here.

Observations indicating that bacterial fusion may occur were reported, during the discussions, by Lederberg and Tatum. Because their report contains new experimental details, it is published in this volume as a separate paper. The report made during discussions by Dr. Arthur Shapiro is also published as a separate paper, since it contains material that can be handled more simply in this way than in the form of a discussion.

At this year's meeting the discussions were so extensive that if they had been fully recorded and published the size of the volume would have been doubled. Instead, the system adopted for the 1942 symposium was followed. Discussions were not recorded, but participants were requested to submit manuscripts of their discussions if they felt that their statements had contributed important information.

During the course of the Symposium, a group interested in nutritional studies with microorganisms formulated a set of recommendations for the use of technical terminology employed in their work. These recommendations are published as an Appendix to the volume.

One hundred and thirty-six individuals were registered as attending the meetings. Attendance at the different sessions varied between 70 and 110. On the afternoon of July 9th the entire group went on a picnic to Jones Beach.

M. DEMEREC

# LIST OF THOSE ATTENDING OR PARTICIPATING
# IN THE SYMPOSIUM

ADAMS, M. H., New York University College of Medicine, New York

ALLEN, PAUL J., University of Wisconsin, Madison, Wisconsin

ALTENBURG, EDGAR J., The Rice Institute, Houston, Texas

ANDERSON, THOMAS F., University of Pennsylvania, Philadelphia, Pennsylvania

ARBOGAST, RACHEL, Carnegie Institution of Washington, Cold Spring Harbor, New York

ASHESHOV, IGOR N., University of Western Ontario, London, Ontario

AVERY, OSWALD T., Hospital of the Rockefeller Institute for Medical Research, New York

BAILEY, WILLIAM, JR., Vanderbilt University, Nashville, Tennessee

BATY, WILTON E., Huntington High School, Huntington, New York

BEDWELL, EVELYN M. P., Strangeways Research Laboratory, Cambridge, England

BERNHEIMER, ALAN W., New York University College of Medicine, New York

BERNTON, HORACE W., Harvard University, Cambridge, Massachusetts

BERTIU, J., National Public Health Service, Santiago, Chile

BODENSTEIN, DIETRICH, Edgewood Arsenal, Maryland

BONNER, DAVID, School of Biological Sciences, Stanford University, California. Present address: Osborn Botanical Laboratory, Yale University, New Haven, Connecticut

BROWN, GEORGE B., Sloan-Kettering Institute for Cancer Research, New York

BRYSON, VERNON, Biological Laboratory, Cold Spring Harbor, New York

BUNTING, MARY I., Bethany, Connecticut

BURKHOLDER, PAUL R., Yale University, New Haven, Connecticut

BUSCHKE, WILLIAM H., Johns Hopkins Hospital, Baltimore, Maryland

BUSH, M. T., Vanderbilt University School of Medicine, Nashville, Tennessee

CARLSON, J. GORDON, National Institute of Health, Bethesda, Maryland

CASON, LOUISE, Medical College of Alabama, Birmingham, Alabama

CLAUDE, ALBERT, The Rockefeller Institute for Medical Research, New York

COHEN, SEYMOUR S., University of Pennsylvania, Philadelphia, Pennsylvania

CORNMAN, IVOR, Sloan-Kettering Institute for Cancer Research, New York

DAVIS, BERNARD D., United States Public Health Service, The Rockefeller Institute for Medical Research, New York

DELBRÜCK, MAX, Vanderbilt University, Nashville, Tennessee

DEMEREC, M., Carnegie Institution of Washington, and Biological Laboratory, Cold Spring Harbor, New York

DE TOMASI, J. A., Cold Spring Harbor, New York

DIENES, L., Massachusetts General Hospital, Boston, Massachusetts

DIPPELL, RUTH, Indiana University, Bloomington, Indiana

DOERMANN, A. H., Vanderbilt University, Nashville, Tennessee

DUBOS, RENÉ J., The Rockefeller Institute for Medical Research, New York

DULANEY, E. L., Merck & Company, Rahway, New Jersey

DUBASH, J., Memorial Hospital, New York

DURYEE, WILLIAM R., National Research Council, Washington, D.C.

EMERSON, RALPH, University of California, Berkeley, California

EMMONS, C. W., Division of Infectious Diseases, National Institute of Health, Bethesda, Maryland

EPHRUSSI, BORIS, University of Paris, Paris, France

FANO, U., Carnegie Institution of Washington, Cold Spring Harbor, New York

FOSTER, RUTH, University of Texas, Austin, Texas

FRIEDENWALD, JONAS S., Johns Hopkins Hospital, Baltimore, Maryland

FROLAY, ALEXANDER, Chas. Pfizer & Company, Brooklyn, New York
GABY, W. L., Bristol Laboratories, Syracuse, New York
GAFFRON, HANS, University of Chicago, Chicago, Illinois
GASIĆ, G., Biological Institute, National University of Chile, Santiago, Chile
GEY, GEORGE O., Johns Hopkins Hospital, Baltimore, Maryland
GILES, NORMAN H., JR., Yale University, New Haven, Connecticut
GLASS, BENTLEY, Goucher College, Baltimore, Maryland
GOPAL-AYENGER, A., Barnard Skin & Cancer Hospital, St. Louis, Missouri
HAGFORS, BIRGIT, Washington University, St. Louis, Missouri
HASKINS, CARYL P., Haskins Laboratories, New York
HERRIOTT, ROGER M., The Rockefeller Institute for Medical Research, Princeton, New Jersey
HERSHEY, A. D., Washington University School of Medicine, St. Louis, Missouri
HOLLAENDER, A., Industrial Hygiene Research Laboratory, National Institute of Health, Bethesda,
    Maryland
HOTCHKISS, ROLLIN D., The Rockefeller Institute for Medical Research, New York
HUTNER, S. H., Haskins Laboratories, New York
JOHNSON, GARTH, Ortho Research Foundation, Linden, New Jersey
JOHNSON, T., Dominion Rust Research Laboratory, Winnipeg, Canada
KAMEN, MARTIN D., Washington University, St. Louis, Missouri
KAUFMANN, B. P., Carnegie Institution of Washington, Cold Spring Harbor, New York
KAUFFMANN, F., State Serum Institute, Copenhagen, Denmark
KIDD, JOHN G., Department of Pathology, Cornell University Medical College, and New York Hospital,
    New York
KIMBALL, R. F., Johns Hopkins Hospital, Baltimore, Maryland
KLEIN, MORTON, University of Pennsylvania, Philadelphia, Pennsylvania
KRAMER, M. D., Washington University, St. Louis, Missouri
KUFFERBERG, ALFRED B., Ortho Research Foundation, Linden, New Jersey
LATARJET, RAYMOND, Institut Pasteur, Paris, France
LEDERBERG, JOSHUA, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut
LEVY, MILTON, New York University College of Medicine, New York
LINDEGREN, CARL C., The Henry Shaw School of Botany, Washington University, St. Louis, Missouri
LINDEGREN, GERTRUDE, The Henry Shaw School of Botany, Washington University, St. Louis, Missouri
LURIA, S. E., Carnegie Institution of Washington, Cold Spring Harbor, New York, and Indiana Univer-
    sity, Bloomington, Indiana
LWOFF, ANDRÉ, Institut Pasteur, Paris, France
MCCLINTOCK, BARBARA, Carnegie Institution of Washington, Cold Spring Harbor, New York
MACDOWELL, E. CARLETON, Carnegie Institution of Washington, Cold Spring Harbor, New York
MARGARETTEN, PHYLLIS, Perth Amboy, New Jersey
MAYR, ERNST, The American Museum of Natural History, New York
MCCARTY, MACLYN, Hospital of the Rockefeller Institute for Medical Research, New York
MCCORMICK, NANCY, Carnegie Institution of Washington, Cold Spring Harbor, New York
MELLON, RALPH, Western Pennsylvania Hospital, Pittsburgh, Pennsylvania
MICHAELIS, L., The Rockefeller Institute for Medical Research, New York
MILLER, ELIZABETH, The Rockefeller Institute for Medical Research, New York
MILLER, J. J., Dominion Laboratory of Plant Pathology, St. Catherines, Ontario, Canada
MILLER, RICHARD A., Carnegie Institution of Washington, Cold Spring Harbor, New York
MONOD, J., Institut Pasteur, Paris, France
MUIR, ROBERT D., Bristol Laboratories, Syracuse, New York
ORMSBEE, RICHARD A., Memorial Hospital, New York
PAPPENHEIMER, A. M., JR., New York University, New York

PERKINS, DAVID D., Columbia University, New York
PHILLIPS, FAITH, Vanderbilt University, Nashville, Tennessee
PIRIE, N. W., Rothamsted Experimental Station, Harpenden, England
PITTENDRIGH, C. S., Columbia University, New York
PLOUGH, HAROLD H., Amherst College, Amherst, Massachusetts
PONTECORVO, GUIDO, Department of Genetics, The University, Glasgow, Scotland
PREER, JOHN R., Indiana University, Bloomington, Indiana
RACKER, E., New York University, New York
RANTIEN, JOHN B., Chas. Pfizer & Company, Brooklyn, New York
RASETTI, F., Laval University, Quebec, Canada
REESE, ELWYN T., J. T. Baker Chemical Company, Phillipsburg, New Jersey
REINER, JOHN M., Washington University, St. Louis, Missouri
RHOADES, M. M., Columbia University, New York
RICHARDS, OSCAR W., Research Division, American Optical Company, Buffalo, New York
RIS, HANS, The Rockefeller Institute for Medical Research, New York
RYAN, FRANCIS J., Columbia University, New York
SCHNEIDER, HOWARD, The Rockefeller Institute for Medical Research, New York
SCHNEIDER, LILLIAN K., Columbia University, New York
SHAPIRO, ARTHUR, 30 Schermerhorn Street, Brooklyn, New York
SIGURGEIRSSON, THORBJORN, The Rockefeller Institute for Medical Research, Princeton, New Jersey
SKOOG, FOLKE, Washington University, St. Louis, Missouri
SONNEBORN, T. M., Indiana University, Bloomington, Indiana
SPIEGELMAN, S., Department of Bacteriology, Washington University School of Medicine, St. Louis, Missouri
STANLEY, W. M., The Rockefeller Institute for Medical Research, Princeton, New Jersey
STARR, MORTIMER P., Brooklyn College, Brooklyn, New York
STARR, PHOEBE B., Stanford University, California
STEINITZ, LOTTI, Heyden Chemical Company, Brooklyn, New York
STEPHENS, S. G., Carnegie Institution of Washington, Cold Spring Harbor, New York
STERN, CURT, University of Rochester, Rochester, New York
STOCK, C. C., Memorial Hospital, New York
STREISINGER, GEORGE, Cornell University, Ithaca, New York
SUSSMAN, H., Washington University, St. Louis, Missouri
SWANSON, C. P., Johns Hopkins University, Baltimore, Maryland
SWANSTRON, MARYDA, Biological Laboratory, Cold Spring Harbor, New York
TATUM, E. L., Osborn Botanical Laboratory, Yale University, New Haven, Connecticut
TAYLOR, HARRIET E., The Rockefeller Institute for Medical Research, New York
TAYLOR, PATSEY J., Carnegie Institution of Washington, Cold Spring Harbor, New York
TREXLER, P. C., University of Notre Dame, Notre Dame, Indiana
VAN NIEL, C. B., Hopkins Marine Station, Stanford University, Pacific Grove, California
WHITE, M. J. D., University College, London, England
WITKIN, EVELYN M., Carnegie Institution of Washington, Cold Spring Harbor, New York
WOLLMAN, S. H., Sloan-Kettering Institute for Cancer Research, New York
WOODRUFF, H. B., Merck Company, Inc., Rahway, New Jersey
ZAMENHOFF, S., Columbia University, New York
ZELLE, MAX, National Institute of Health, Bethesda, Maryland
ZIMMER, ESTHER M., Stanford University, California

# CONTENTS

# MORPHOLOGICAL AND CHEMICAL RELATIONS IN VIRUSES AND BACTERIOPHAGES

THOMAS F. ANDERSON

Soon after the first demonstration of a submicroscopic disease-producing agent by Iwanowski in 1892, it was realized that many such agents exist. In those early, groping days all obligate intracellular parasites which could be shown to produce a disease in at least one type of cell and which in the free state were smaller than most bacteria were lumped together under the collective term "virus." The viruses were named according to the original host and the disease symptom produced. This nomenclature still persists, mainly because of our continuing ignorance of enough properties of the several viruses to make a logical attempt at classification.

In the past ten years the application of the techniques of general and biological chemistry, genetics, and physics has given us some rather concrete conceptions of a few of the kinds of things we call viruses. These researches, directed mainly at characterizing the free infectious agents or virus particles, have shown that they constitute an extremely heterogeneous group and have enabled us to recognize certain similarities and dissimilarities among the various members. What these results mean in regard to the classification of the viruses is for the future to decide.

At the start it must be recognized that the viruses have a life cycle which can be roughly divided into three parts:

(1) A stage of attack or specific adsorption on a susceptible host cell (probably followed by penetration into the cell).

(2) A stage of development, involving the eventual multiplication of the virus within the cell. Finally, upon maturing, many new particles are liberated from the host, to enter the third stage.

(3) A dormant period of inactivity, in which the free virus particle awaits contact with another susceptible host cell.

While the virus particles are in the last, dormant stage they present easy objects for study, for then many of them are relatively stable, like the seeds and spores of higher forms. We can analyze them chemically, concentrate and study them in the centrifuge, and determine their shapes in the electron microscope, provided only that we can be certain that we are indeed examining the infectious agent and not some innocuous set of particles of debris from the host. It should be clearly understood that attempting thus to classify viruses on the basis of the morphology, or chemical composition, of the infectious particle may be just as difficult and misleading as attempting to classify plants, bacteria, or fungi solely on the basis of the size, structure, and composition of their seeds or spores. On such a basis it might be difficult to tell that the date and the coconut, for example, come from two rather similar plants. What is obviously needed for purposes of classification is a clearer understanding of the processes that occur in the vegetative stages of the viruses.

In the present title, the word "relation" shall thus refer to any aspect of the virus problem that might throw light on the nature of these agents. Logically, it is only by seeing the relations between two or more observations that our minds can grasp the significance of an experience. Redundancy in using both adjectives, "morphological" and "chemical," in reference to objects as small as the viruses may be necessary until it is generally recognized that morphology on an atomic scale is chemistry.

It seems desirable to group the viruses with respect to host—bacterial, plant, or animal—for the purposes of this discussion. This is because of the largely accidental (from the point of view of the virus) but variable factors of: (1) the ease of separation and discrimination of virus particles from the debris of various types of host cell; and (2) the kinds of experiments that have been done with the several viruses, as influenced by the nature of the host and by the interests, objectives, and tastes of the experimenters in the several fields. Naturally, the present review cannot pretend to be a complete account of all pertinent data, but reflects the author's interests. The electron microscope, which plays a large part in the direct determination of virus morphology, was described at these meetings a few years ago (62, see also 61).

## THE BACTERIAL VIRUSES

Of all the viruses, some of the bacterial viruses are the best identified. In 1941 H. Ruska (41) reported the presence in electron micrographs of club-shaped objects resulting from lysis of bacteria by phage, and later he (42) and Kottmann (28) described various forms—clubs, spheres, and rods—which Ruska summarized in an extensive review (43). Apparently, the German workers first studied lysis by using preparations of mixed phages and so were uncertain whether the various structures they pictured were really different stages in the life history of one type of "phage" or merely interesting particles of bacterial debris. These uncertainties were largely removed in the later work, in which lysis by single strains of phage was studied.

## The Bacteriophage Particles of the T Set Active on E. coli Strain B

Meanwhile, we (32, 34) studied unmixed strains of T1 and T2 phages with the electron microscope in this country and were "inclined to identify the particles described . . . with the actual particles of bacteriophage for the following reasons: (a) they are always present in highly active phage suspensions and missing in any control suspensions (media, bacterial cultures, bacterial filtrates, etc.); (b) they are readily adsorbed by the bacteria; (c) the size from a given strain is uniform and corresponds essentially to measurements by indirect methods; (d) the structure of both the 'head' and the 'tail' is characteristic of the strain of phage; (e) preliminary experiments on the lytic process seem to demonstrate the liberation of these particles from the lysing bacteria." Such criteria as these should be applied in the identification not only of phage particles but of other viruses as well.

This work has been extended (5, 17) to each of the seven viruses in the original T set investigated genetically by Demerec and Fano (18). The results have shown that these viruses fall into four morphological groups:

(1) *Virus T1* (Fig. 1) consists of a 500 A round, dense head to which is attached a tail some 1200 A long and about 100 A in diameter.

(2) *Virus T5* (Fig. 2) consists of a 900 A round head of low density with a tail 1700 A long and perhaps 150 A in diameter, attached like a short string to a balloon. The low density of the head in conventional micrographs (5) and the shape of the shadow which it casts in preparations on which gold has been obliquely evaporated suggest that in suspension the head contains considerable amounts of water, which escape on drying in the high vacuum, to leave behind the collapsed head structure observed.

(3) *Viruses T3 and T7* (Fig. 3) are both small spheres about 450 A in diameter, with no visible tails in the electron micrographs. The apparent low electron density suggests that they may be flat or that there may be some water of hydration of the particles in solution. T3 and T7 belong to the same serological group.

(4) *Viruses T2, T4, and T6* have similar, but interesting, complex morphologies. Each has a head 600 × 800 A, frequently pointed, containing a dense internal structure, and a well-defined tail about 1000 A × 200 A, which is frequently parallel to a discernible axis of symmetry of the structure of the head (Fig. 4). Electron micrographs (Fig. 5) of preparations which have been dried and then shadowed with gold show that, after drying, these viruses have become flattened; for their shadows are not so long as those that would be cast by spherical particles, nor do they have the expected shapes. These observations argue strongly for the idea that the active particles in aqueous suspension have water within them.

Unfortunately, it is somewhat difficult at present to estimate just how much water is incorporated in virus particles from an analysis of the shapes of the shadows they cast; for if one shadows from below a thin membrane supporting a number of virus particles, one finds, as shown in Fig. 6, that the membrane has been distorted by the virus particles on the other side. The mechanism by which this effect is produced has not been worked out. Presumably, in the last moments of drying the specimen for observation in the electron microscope, the surface tension of the liquid around the particles forces them into the supporting membrane. The distortion thus produced in the membrane is at least partially retained after all the liquid has evaporated. In any event two factors contribute to the shape of the shadow cast by a virus particle: (1) the shape of the dried particle and (2) the extent to which the particle has distorted the supporting membrane. If the proposed mechanism is correct, factor (2) would be determined by a number of variables, many of which are difficult to control: (a) the surface tension of the virus suspension, (b) the tautness and rigidity of the supporting membrane, (c) the rigidity, shape, and size of the virus particle, and (d) the extent to which the distortion in the membrane is retained after all the water has evaporated from the specimen. Since the possibility of plastic flow of the virus particle and the membrane may be involved, the *rate* of evaporation of the water may also be a contributing factor.

Further information regarding the structures of these viruses has been obtained by treatment with various physical or chemical agents. For example, when T2 is subjected to sonic vibration, dilute NaOH, or intense ultraviolet irradiation, it is broken up and "ghosts" of the virus particles are left behind, as shown in Fig. 7. Apparently these treatments allow the internal structure to escape from the heads of the particles and leave behind the empty membranes, which surrounded the internal structure and to which the tail is still attached. Similar ghosts have been seen in untreated preparations of T2, T4, and T6; and some old preparations contain many free tails with no recognizable trace of the heads attached. Thus the gross morphologies of these even-numbered viruses are very similar.

Antisera prepared against virus T2 have been found (17) to inactivate not only virus T2 but viruses T4 and T6 as well. Such an antiserum does not react with any of the other viruses of the T set. One therefore speaks of viruses T2, T4, and T6 as belonging to the same serological group. Likewise, antiserum against virus T3 reacts with viruses T3 and T7 only; they belong to another serological group. Antiserum against T1 reacts only with T1; antiserum against T5 reacts only with T5. T1 therefore occupies a separate serological group; T5 oc-

cupies another. Recent work on the specificity of antigen-antibody reactions has indicated that a hapten will react strongly with an antibody only if its structure is nearly congruent (within a few tenths of an angstrom) to that of the hapten which, in combination with a protein, elicited the formation of the antibody in question (37). It would follow from their serological reactions that the similarity in some antigenic parts of viruses T2, T4, and T6 extends down to the atomic scale. T3 and T7 are likewise similar to each other in this respect.

Chemical analysis of T2 and T4 from bacteria grown on an ammonium-lactate medium shows that essentially all the phosphorus exists in combination with desoxyribose nucleic acid, of which T2 contains 37% (14, 58). Chemical analyses of the other viruses in the T set have not yet been reported.

### Other Bacteriophage Particles

A number of phages active on hosts other than *E. coli* strain B have been studied in this country. One of them, active on a strain of *Pseudomanas pyocyanea*, has been found (45) to resemble T2, T4, and T6 in morphology, although it may be somewhat larger. Another phage active on a strain of Staphylococcus has a head about 1000 A in diameter and a tail about 2000 A long (34). In general appearance it resembles T5. A third phage, P2, active on a motile strain of *E. coli* is round, 500-600 A in diameter, with no visible tail (34).

A number of phages studied by German workers during the war have morphologies that are strikingly similar to those found in this country. However, others have been reported to have large diplococcus-shaped heads; and Kottman (28) has pictured phage particles shaped like tiny bacilli, 2000 A or so long and about 400 A wide. Unfortunately, much of this work was carried out with mixed phages, so that the workers were originally led to consider the various forms observed as different stages in development of "d'Herellen" as Ruska terms them. Ruska reports that the tadpole-shaped "d'Herellen" adsorb tail first on the host (Fig. 8), while Kottmann's bacillus-shaped phages orient themselves perpendicular to the surface of the host (*E. coli*). One of his micrographs, Fig. 9, shows about 140 such particles closely packed around the periphery of the host cell, suggesting either that these phages are adsorbed in a narrow band, which circumscribes the host cell, or that on drying they are compelled by surface-tension forces to take up positions about the equator of the drying cell. This raises the interesting question whether there may not be a narrow zone or strip on the surfaces of certain bacteria on which some phages are preferentially adsorbed.

In general, as originally discoverd by filtration data and later by inactivation volumes determined by X-rays, the sizes of the phages active on a given organism have been found to be inversely related to the sizes of the plaques that they produce on the host smear.

The many bacterial viruses are distinguishable from one another in that they have different host ranges. For example, with the possible exception of T3, which forms large plaques, and T4, which forms small plaques, all the viruses of the T set of Demerec and Fano have different sets of hosts among the various mutant strains of *E. coli* B. As we learn more about the viruses, new differentiating criteria will doubtless emerge.

### The Activation of T4 and T6 by Aromatic Amino Acids

A new type of character has been found to differentiate T4 and T6 from the other viruses of the T set. Unlike preparations of the others, most particles in preparations of T4 and T6 do not readily form plaques on the host in a synthetic ammonium-lactate medium, although this medium adequately supports growth of the host (2). Since T4 and T6 readily form plaques on Difco nutrient medium prepared from biological sources, it appeared that some substances present in this medium promote the activity of these viruses. Consequently, a great number of the growth factors and amino acids known to be present in the Difco medium were tested individually by plating the virus with the host in the presence of the compound in question. Of all the compounds tested in this way, *l*-tryptophane was found to be the most active, while phenylalanine, diiodotyrosine, and tyrosine showed progressively lower activities. Compounds that promote the activity of virus on their host have been termed virus cofactors (2).

When samples from a mixture of T4 and the host bacteria in the synthetic medium were examined in the electron microscope, it was found that the virus was not adsorbed on the host. When tryptophane was added to such a mixture, however, the virus was found to be adsorbed. It follows that tryptophane promotes the adsorption of this virus on the host.

That tryptophane activates the virus rather than the host is shown by the results of the following two experiments. In the first experiment, T4 was incubated with 20 gamma *l*-tryptophane/ml. for 30 min., after which 0.1 ml. was added to 10 ml. of a suspension of the host. It was found that 50% of the virus was adsorbed (as determined by centrifugation of the bacteria) and formed plaques when plated on ammonium-lactate agar. In the second experiment, the host bacteria were incubated with 20 gamma of *l*-tryptophane/ml.; a 0.1-cc. sample was then added to 10 ml. of virus to give a suspension of the same composition as the second tube of the first experiment. No detectable amount of adsorbed virus was found in the second experiment, nor did the virus form plaques. The virus cofactor

tryptophane, therefore, activates the virus rather than the host. All attempts to detect a corresponding activation of the bacteria have been unsuccessful. Incidentally, the above experiment also shows that the virus T4 is not activated appreciably by concentrations of *l*-tryptophane below 0.2 gamma/ml.

Additional experiments (*3*) have shown that the reaction between virus and tryptophane may be looked upon as being reversible. For example, when the tryptophane concentration is decreased by dilution of a suspension of T4 in tryptophane, the activation of the virus disappears in a rapid reaction which appears to be of first order. At room temperature only about 0.1% of the original activation remains 60 seconds after such a dilution is made. Studies of the temperature dependence show that the percentage of activation by 20 gamma *l*-tryptophane/ml. is maximum at about 35° C. At room temperature this activation has a broad maximum at about pH 7.5.

The mechanism of adsorption of a virus particle on its host cell is still a matter of uncertainty. The view most generally held is that the bacterium has on its surface a number of receptive spots whose configuration is complementary to that of a part of the surface of the virus particle, much as an antigen is supposed to have a structure complementary to that of its antibody. Van der Waals forces and other forces are then supposed to hold them together when they come in contact. Another theory is that incorporated in the virus is a type of enzyme whose specific substrate is part of the bacterial cell wall. In the cofactor effect it seems possible that tryptophane acts by altering the surface of the virus particle to fit the shape of the host's receptor spot for T4. Or the cofactor may act as a type of loosely bound coenzyme or prosthetic group for that component of the virus particle which is responsible for its entry into the host when by chance it encounters such a cell in the medium. Naturally, these two possibilities are not mutually exclusive.

Further clues to the mechanism of action of the cofactors may be gained by inspection of the structures of certain compounds whose activities have been determined (*4*). Such tests of activity were made on a number of tryptophane analogues and derivatives, which were obtained through the kindness of Drs. R. W. Jackson and W. G. Gordon of the Eastern Regional Research Laboratory of the U. S. Department of Agriculture. These tests showed that any alteration of the α-amino group of tryptophane by removal or by substitution of one of the hydrogens resulted in loss of all measurable activity. Likewise, reduction of the carboxyl group of tryptophane to form 3-indolepropanolamine, or removal of the carboxyl group altogether, destroyed the activity.

The question of which stereo-isomer, *d* or *l*, is the active form of tryptophane was solved through the kindness of Dr. C. P. Berg of the State University of Iowa, who provided a purified sample of *d*-tryptophane. The sample exhibited but 1% of the activity of pure *l*-tryptophane, in agreement with the proportion of *l*-tryptophane estimated to be present from the specific optical rotation of the sample. From these results it is to be concluded that *d*-tryptophane is inactive and that the reaction leading to activation of T4 virus specifically requires an *l*-α-amino-acid grouping.

The other structural elements that the active cofactors have in common can be seen from the list given in Table 1. The naturally occurring aromatic amino acids phenylalanine, tyrosine, and diiodotyrosine were active; but thyroxine, in which the phenolic hydrogen of diiodotyrosine is replaced by another diiodophenol group, is inactive, presumably because the aromatic group is too large. 2-methyl and 5-methyltryptophane have rather high activities. The latter is bacteriostatic in the absence of tryptophane, evidence that the reaction which it blocks in the host may not be involved in the initial stages of the virus's attack on the host. The detoxication product *p*-bromphenylcysteine and the related *p*-brombenzylcysteine are active and show no unusual effects.

The last two cofactors listed, 2- and 3-pyridylalanine, are particularly interesting, for at suitable concentrations they permit growth of the host but inhibit the formation of plaques by T4. This indicates that they are capable of blocking, more or less specifically, some reaction involved in the multiplication of T4 virus. This, of course, is exactly what is required of a chemotherapeutic agent against viruses. This phenomenon should encourage us to look for cofactors in virus diseases of higher organisms and to test the effects of analogues of the active substances in blocking virus multiplication.

Incidentally, it might be well to point out here that the cofactor phenomenon may be of wide significance to cytochemistry in general. The mechanisms of coordinated movements of intracellular

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EXPLANATION OF FIGURES ON OPPOSITE PAGE

Fig. 1. Bacteriophage T1 (*34*). Negative 789e. 36,000 ×. (Courtesy of the Journal of Bacteriology.)

Fig. 2. Bacteriophage T5, shadowcast with a 7-to-10 A film of gold. Negative 202b. 36,000 ×.

Fig. 3. Bacteriophage T3 adsorbed on *E. coli* strain B and shadowed with gold. Negative 246c. 36,000 ×.

Fig. 4. Bacteriophage T6 in a conventional electron micrograph. Note the ghost in the lower part of the field. Negative 162e. 21,000 ×.

Fig. 5. Bacteriophage T4 shadowed with gold. Negative 199b. 36,000 ×.

Fig. 6. A formvar specimen-supporting membrane with T4 particles on the reverse side, shadowcast with gold to show the deformation of the membrane by the virus particles. Negative 223d. 22,000 ×.
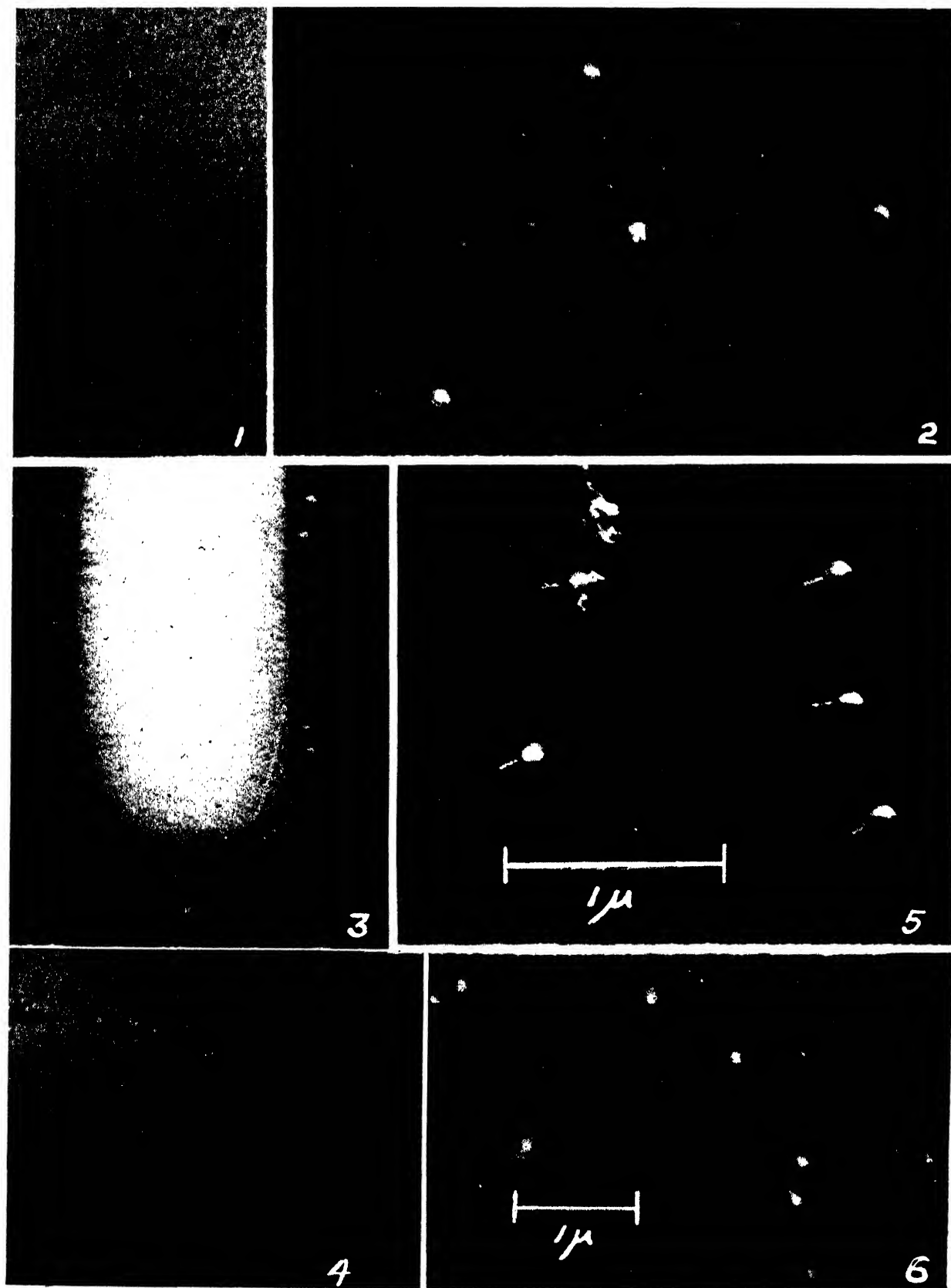
FIGS. 1-6 (see facing text page for legends).
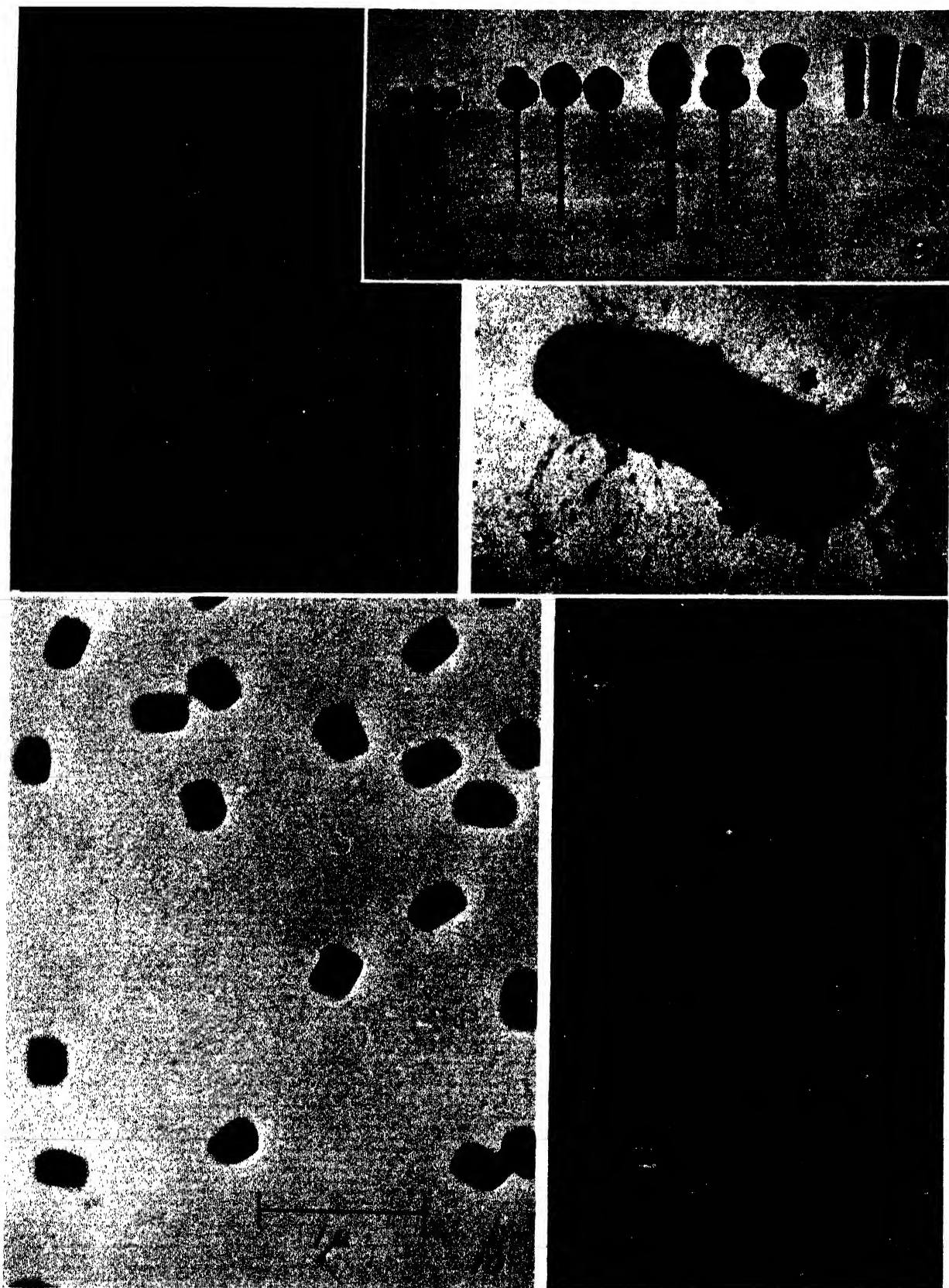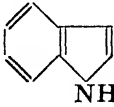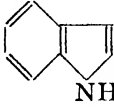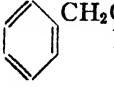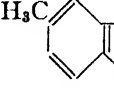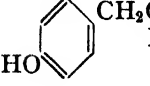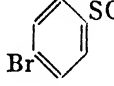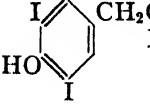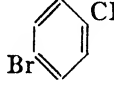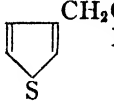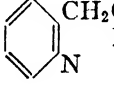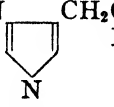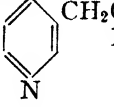
FIGS. 7-11 (see facing text page for legends).

constituents—such as chromosomes in pairing, spindle fibers in mitosis, and myosin fibrils in muscle contraction—have long been a mystery. One theory of such activity postulates the intracellular release, on demand, of specific compounds whose

reactions, the antigen-antibody combination is a special case in which the specific antibody protein generally has two or more "valences" which bind similar antigen molecules in this way. The cofactor effect is the first instance, to my knowledge, in

### TABLE 1. COFACTORS FOR T4 AND T6 VIRUSES

| Compound | Approximate Activity for T4 Virus | Compound | Approximate Activity for T4 Virus |
|---|---|---|---|
| (indole) $CH_2CHCOOH$ $NH_2$ / NH | $l = 100$ $d = 0^{****}$ | (indole) $CH_2CHCOOH$ $NH_2$ $CH_3$ / NH | $dl = 10^{*}$ |
| (benzene) $CH_2CHCOOH$ $NH_2$ | $dl = 1$ | $H_3C$ (indole) $CH_2CHCOOH$ $NH_2$ / NH | $dl = 10^{*}$ |
| $HO$ (benzene) $CH_2CHCOOH$ $NH_2$ | $dl = 0.1$ | $Br$ (benzene) $SCH_2CHCOOH$ $NH_2$ | $dl = 1^{*}$ |
| $I$ $HO$ $I$ (benzene) $CH_2CHCOOH$ $NH_2$ | $dl = 1^{*}$ | $Br$ (benzene) $CH_2SCH_2CHCOOH$ $NH_2$ | $l = 1^{**}$ |
| (thiophene, S) $CH_2CHCOOH$ $NH_2$ | $dl = 1^{**}$ | (pyridine, N) $CH_2CHCOOH$ $NH_2$ | $dl = 1^{***}$ |
| $N$ $N$ $CH_2CHCOOH$ $NH_2$ | $dl = 0$ | (pyridine, N) $CH_2CHCOOH$ $NH_2$ | $dl = 1^{***}$ |

    * Gift of W. G. Gordon and R. W. Jackson
   ** Gift of V. du Vigneaud
  *** Gift of C. Niemann
**** Gift of C. P. Berg

bivalent affinities for the structural elements involved cement them together when thermal agitation brings them in contact. Among extracellular

which relatively simple compounds somehow are able to promote the specific combination of two functionally distinct biological units.

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### EXPLANATION OF FIGURES ON OPPOSITE PAGE

FIG. 7. Ghosts of T2 bacteriophage produced by intense ultraviolet irradiation. Negative 132b. 24,000 ×.

FIG. 8. Sketches of various types of morphology of "d'Herellen" as seen in the electron microscope by H. Ruska and other German workers (43). 100,000 ×.

FIG. 9. The bacillus-shaped bacteriophages adsorbed on the cell wall of their host, E. coli, as observed by Kottmann (28).

FIG. 10. Vaccinia virus elementary bodies (21). Negative 442c. 28,000 ×.

FIG. 11. Vaccinia virus after treatment with dilute NaOH, showing the external membranes of the elementary bodies from which the internal structure has escaped (21). Negative 448c. 28,000 ×.

## Degradative Analysis of Physiological Properties

In unraveling the structure of a complex organic compound having a certain set of properties, the chemist degrades it by successive treatments with heat, acid, alkali, etc., to split off identifiable component parts from a residue which then has a new set of properties, some of which are related to those of the original material. From a knowledge of the parts split off and the order in which the original properties disappeared and new ones appeared in the residues, the chemist attempts to visualize the reversal of the stages in the degradations and thus to suggest syntheses that confirm or disprove his mental reconstruction of the initial compound.

Similarly, the progressive degradation of a virus eliminates in succession the various physiological properties that are characteristic of the original virus. The results of such an indirect attack, serving to relate various physiological activities to one another, will help to elucidate the interplay of the several structural details that determine the interactions of the virus with its host. In a number of cases it has been possible physically to separate by various treatments components that exhibit at least one of the physiological activities characteristic of the intact virus.

An example of this type of attack is seen in the effects of ultraviolet light on the bacterial virus T2. Suspensions of T2 that have lost all but 0.001% of the initial plaque-forming activity as a result of such irradiation, when added to host cells, interfere with the multiplication of fresh T2 virus and of other T viruses added later to the host cells (33). It is interesting that the few virus particles that survive such treatment have delayed rates of growth on susceptible host cells (31).

T2 exhibits another activity, that of summarily lysing heavily irradiated host cells without phage multiplication (1). This activity appears to be separable from the main body of the virus particle. As irradiation with an intense arc is continued to reach doses far beyond those used in the experiments discussed in the previous paragraph, this lytic activity of a T2 suspension rises to a maximum and then falls to nil as the turbidity decreases markedly and the viscosity of the suspension increases. Electron-microscope pictures of such suspensions show, as might be anticipated from the last two observations, that the virus particles have been decomposed by the treatment into empty-headed ghosts and debris. If the fractions receiving various degrees of treatment are now centrifuged at a speed (30,000 r.p.m.) sufficient to throw down most of the intact virus, it is found that the supernatant liquids contain amounts of lytic activity which rise to a maximum at the point of maximum activity of the irradiated but uncentrifuged suspensions. The activity then decreases as irradiation is continued. These results indicate that ultraviolet irradiation frees lytic activity from the major fraction of T2 particles, but at the same time inactivates it as it would an enzyme. Whether the activity resides in the ghosts or in still smaller components has not yet been determined. Taken altogether, the results of ultraviolet treatment of T2 virus (and of influenza virus discussed later) indicate that the complexity of its physiological composition parallels its apparent morphological complexity.

## Résumé

(a) The structural characteristics of the T set of viruses place them in four morphological groups, which agree with the serological classification. The classification thus arrived at bears no obvious relation to classifications based on similarity between host ranges among mutant strains of the host, E. coli B.

(b) On a "complete" medium the "infectious ratio," or the ratio of the number of plaques (lesions) produced on a bacterial smear (tissue) to the number of virus particles placed on the smear, is very nearly unity; but on a synthetic medium the ratio for most preparations of two of the viruses, T4 and T6, is only 0.001 or less. A trace of an aromatic amino acid restores these two viruses to almost complete activity.

(c) The various physiological activities (infectivity, interference, summary lysis, etc.) that T2 virus exerts on the host can be degraded in separable steps.

The fact that one cannot immediately generalize these phenomena in passing to animal and plant viruses should not deter us from looking for analogous things in the latter material.

## ANIMAL VIRUSES

Work with most animal viruses is handicapped by three factors. First, normal cells of animals contain particles (mitochondria, granules, etc.) whose sizes overlap the range of virus sizes (12, 39); it is therefore difficult to separate some of the animal viruses from normal particles by such means as differential sedimentation or filtration.

Second, in most animal viruses even the best preparations give low infectious ratios, of the order of 0.001 or less. This may be interpreted as indicating that the preparations are impure, that the route of infection or the virus-host interaction itself is inherently inefficient, that the host has a defense mechanism which protects it from infection, or that unknown but essential substances like cofactors are inadvertently omitted from the viruses in preparation and application to the host. The current practice of suspending virus in buffered saline during purification and dilution for assay obviously bears on the last point. If no more than one in a thousand of the particles in a preparation can be shown to be capable of producing a lesion, one cannot be sure that any particular object seen in an electron micrograph is the infectious one. In

bacterial infections, too, a thousand or more organisms are frequently required to produce disease. But, since the bacterial cell can multiply outside the host, it can usually be shown that the thousand offspring of a single cell or two, grown outside the animal, can produce the disease. In virus diseases, of course, we do not have this method of studying the potentialities of a single unit, but can study only those of an inoculum of a thousand particles or more. It follows that, in describing a virus preparation as seen in the electron microscope or studied in the ultracentrifuge, one is rightly forced to use the expression "activity is associated with particles" having a particular morphology or sedimentation constant, rather than saying that "the active virus particle" has such and such a morphology or rate of sedimentation.

The third difficulty is related to the complexity of the host, with respect both to the cellular make-up of its tissues and to the unknown and (at present) largely uncontrollable nutrient body fluids in which its cells are bathed. An animal host must be looked at from the ecological point of view (8) as a closely knit community of cooperating cells, only a fraction of which is attacked by the virus, while others in the community may attempt to ward off the attack. It is accordingly difficult to carry out direct biochemical studies on the reproduction of the animal viruses.

On the other hand, it need hardly be mentioned that some complex aspects of the response of the animal host—namely, the immunological reactions and the hemagglutination reaction (24)—provide the investigator with powerful tools for virus study.

Another advantage to the worker in the field of animal viruses is the fact that man, recognizing his kinship to the animals, has provided more adequate funds for this work. The public also provides a powerful incentive to these researchers in its ready appreciation of the advances made in this field, while in general it has great difficulty appreciating the perhaps more fundamental results of studies on the growth of bacterial or plant viruses. The long-range wisdom of an exclusively frontal attack, however, does not appear so obvious as it did, say, ten years ago.

There is a huge literature on animal viruses and virus diseases (cf. 8), but here we can be concerned only with those viruses that have been at least partially purified and whose morphologies have been investigated.

### The Elementary Bodies

Of all the animal viruses, the elementary bodies of vaccinia are perhaps the best identified. Smadel, Rivers, and Pickels (51) obtained infectious ratios between 0.4 and 0.1 for their best preparations. Electron micrographs of such preparations show (Fig. 10) that they contain particles of rather uniform aspect, brick-shaped, $0.2 \times 0.3$ $\mu$. Most of them can be seen to contain five regions of higher density arranged like the 5 spots on the faces of dice (21), a structure which is brought out more clearly in shadowed preparations (49). When treated with dilute NaOH, the internal structure escapes from the particles and the resulting "ghosts" (Fig. 11) appear to be the remains of the membranes of the original particles (21). Apparently the impermeability of the membranes to such substances as sucrose is responsible for their behaving as osmometers (50). Their organization is thus analogous to that of bacteria (36) and to the heads of T2 bacteriophage previously mentioned.

Ruska and Kausche (44) have made statistical studies of the sizes and shapes of 5 types of elementary body, with the results given in Table 2. The dimensions of individual particles gave a Gaussian distribution about a mean value for length, width, and the ratio between the two. These results contrasted strongly with the distributions of lengths and widths of bacterial cells in a growing culture of Staphylococcus. Because such cultures contained many cells that were about to divide, the distribution of cell sizes deviated strongly from the normal Gaussian distribution, particularly on the large side of the means. These authors conclude that elementary bodies probably do not multiply by a simple process of growth and division; or that, if they do, the process has somehow stopped, so that a Gaussian size distribution results by the time the preparations have been extracted and purified.

TABLE 2. THE SIZES OF VIRUS ELEMENTARY BODIES (44)

| Virus | Arithmetic Mean and Mean Deviation $\sigma$ | | |
|---|---|---|---|
| | Length, m$\mu$ | Breadth, m$\mu$ | Axial Ratio |
| | (a)　　($\sigma$) | (b)　　($\sigma$) | (q = a/b)　($\sigma$) |
| Mollusc contag. | $255 \pm 61.3$ | $178 \pm 41.8$ | $1.45 \pm 0.143$ |
| Ectromelia | $232 \pm 24.6$ | $172 \pm 9.6$ | $1.34 \pm 0.024$ |
| Myxoma | $287 \pm 20.7$ | $233 \pm 19.0$ | $1.22 \pm 0.122$ |
| Canary pox | $311 \pm 26.1$ | $263 \pm 22.7$ | $1.14 \pm 0.180$ |
| Vaccinia | $262 \pm 28.2$ | $209 \pm 23.9$ | $1.25 \pm 0.124$ |

### Influenza Virus

Taylor *et al.* (*59*) have reported that active preparations of influenza virus PR8 contain spheres and kidney-shaped particles about 800 A in diameter, in agreement, as far as size is concerned, with earlier results based on differential filtration through graded membranes. This work has been confirmed in extensive studies by Stanley (*54*), Knight (*26*), and others (*29*), who have reported particles about 1000 A in diameter with an estimated density of 1.1. Each would then weigh approximately $6 \times 10^{-16}$ gm. The most active preparations obtained by Knight (*26*) gave 50% infectivity end-points in chick embryos at $10^{-12}$ to $5 \times 10^{-15}$ gm. The infectious ratios for these preparations would therefore appear to be in the range of

$$\frac{1}{2} \times \frac{6 \times 10^{-16}}{10^{-12} \text{ to } 5 \times 10^{-15}} = 0.0003 \text{ to } 0.06$$

Ratios about 1/100th of these may be calculated from the observed infectivity of the preparations for mice. The behavior of the particles during sedimentation in sucrose solutions of different densities suggests (*29*) that they contain about 60% by weight of water. Indeed, to the author the low densities of the majority of electron-microscope images obtained without staining suggest that most of the particles are like empty bags, which have collapsed on drying. On the other hand, the gold-shadowed preparations of Williams and Wyckoff (*60*) and those treated with .023 M CaCl₂ by Taylor *et al.* (*59*) contain many particles that appear relatively solid and rounded. In view of the rather low infectious ratios, it is somewhat difficult to say at this time which of the various morphological types in this size range is the actual infectious agent.

The situation is further complicated by the fact that the particles of the PR8 and Lee strains of influenza contain a minimum of 20% and 30%, respectively, of antigen inseparable from the activity but characteristic of the normal tissue particles of the host on which the virus was grown (*13, 27*). While, conventionally, this "extraneous" antigen would be considered to be an impurity, Cohen (*13*) points out that at the active or proliferating stage in the virus's life cycle such a close association between virus and host material might be expected. On the other hand, when the nature of the infectious agent varies in this way with the host on which it grows, it becomes difficult to characterize that agent as a unique entity.

A somewhat analogous situation has recently been resolved by Horsfall and Curnen (*25*) in the case of the pneumonia virus of mice, which occurs in many laboratory animals and humans. The infectious particle extractable from infected lungs is 1000 to 1500 A in diameter and consists of the virus particle in combination with a component of normal tissue. Appropriate treatment with heat or alkali, however, splits the virus hemagglutination activity apart from the normal component. Similiarly, on appropriate extraction from infected lung tissue the infectivity is found to be associated with relatively small particles 250 to 300 A in diameter. So far, the infectivity of influenza virus has not been separable from normal antigens, although the results of an earlier study (*10, 11*) seemed to show that 100 A particles carried the infectivity. On the other hand, one of the complement-fixing antigens of influenza is not sedimentable at 90,000 g (*23*).

The Henles (*22*) have shown, however, that like those of T2 phage the various physiological activities of influenza virus preparations can be separately degraded by ultraviolet irradiation. Irradiation quickly destroys the infectivity, while the toxicity and immunizing capacity are much more slowly inactivated. Long periods of irradiation fail to affect the hemagglutination appreciably, until a point is reached when this activity rather suddenly disappears. Similarly, long periods are required to destroy the ability of the material to fix complement in combining with antibody. The apparent ability of the virus to interfere with active virus of the PR8 strain rises to a maximum only after essentially all the infectivity has disappeared. This activity remains at a high level for a while and is eventually destroyed as the irradiation is continued. The fact that the various activities are destroyed at different rates indicates that either different atomic groups in the particle (having different sensitivities to the radiation) or different residues of organization of the native structure of the active particle as a whole are required for the expression of each of the several activities.

### Other Animal Viruses

A number of animal viruses have been purified and examined in some detail in the centrifuge and the electron microscope in an attempt to identify infectivities with particles having characteristic sedimentation constants and morphologies. In most cases, however, the infectious ratios have not been given; or, where they have, they have been rather lower even than the highest reported for influenza virus. Until the infectious ratios can be brought nearer to 1, the best that can be done with regard to morphology is to report the structures of the majority of particles in the cleanest preparations.

Thus the Eastern and Western strains of equine encephalomyelitis are reported (*47*) to be spheres or discs approximately 400 A in diameter. The electron-microscope studies show (*46, 48*) the dried protein of rabbit papilloma virus to consist of spheres or discs about 440 A in diameter, while studies combining centrifuge and diffusion techniques indicated a value of 660 A. Like vaccinia and influenza virus, this virus changes its density in solutions of low-molecular-weight materials, which suggests either the penetration of solute into the particle or the osmotic removal of water from it. Bovine serum albumin as a solute, however, does not

produce this effect, permitting the direct estimation of its density (1.13) as compared with that of influenza virus (1.10). The virus contains 1.04 gm. water to 1.0 gm. of dry virus protein.

The morphology of the virus of poliomyelitis seems to be an uncertain matter. Gard (*19*) reports that procedures which concentrate the virus activity in infective tissue preparations concentrate rods 150 × 1150 A. The same procedures applied to non-infectious material concentrate similar rods. In contrast, Loring and Schwerdt report (*30*) that the concentration of activity of the Lansing strain of poliomyelitis leads to preparations containing spheres which are small as seen in the electron microscope and which have sedimentation rates of 82 ± 8 S.

Finally, mention should be made of studies on silkworm jaundice, sometimes termed polyhedral disease because of the crystal-shaped inclusion bodies that are found in the nuclei of infected insects. While Glaser and Stanley (*20*) found the inclusion bodies to be infectious, they were unable to liberate active material from them. On the other hand, the concentration of the active principle from blood of infected silkworms to the point where $10^{-12}$ gm. of material per cc. was active, yielded particles 100 A in diameter with sedimentation rates of 17 S. Nevertheless, $10^{-12}$ gm. of such material would contain about 2,000,000 particles, a large proportion of which might well be the component derived from the blood of normal silkworms, which, these authors point out, has a sedimentation constant of 16 S.

## PLANT VIRUSES

Work with plant viruses has numerous advantages, not the least of which is the fact that the plant cell contains relatively small numbers of particles in the general range of virus sizes. Infection of plant cells with a suitable virus induces the formation within the cells of relatively huge amounts of protein particles rather homogeneous in characteristics. The properties vary with the strain of infecting virus, and the purification of these particles is usually accompanied by an increase in the specific infectivity over that of the extracted juice of the infected plant. The evidence is therefore strongly in favor of the view that infectivity resides in these protein-containing particles.

Since a number of excellent reviews of the status of the problem of the plant-virus particle have recently appeared (*6, 57*), it seems desirable to present only a few of the more salient points here, together with some data that have only recently become available.

As everyone knows by now, viruses in this group, whose physical properties have been studied in detail, form "crystals." Ordinarily the term "crystal" would imply that the particles are packed together in a solid structure, "bounded by plane surfaces symmetrically arranged, which are the external ex-

pression of a definite internal structure." Actually, tobacco mosaic virus, the most famous of these viruses, forms "crystals" bounded by *curved* surfaces; the rods in which its activity must reside are packed hexagonally side by side, but without much if any tendency for the ends of the rods to be regularly arranged with respect to one another (7). These have been termed "two-dimensional crystals," since the arrangement is regular in one plane only.

The spherical plant viruses form "crystals" bounded by plane surfaces. Even here, however, it is doubtful if these structures are crystals in the strict sense. An implicit assumption of a great deal of plant-virus work has been that the activity of the particle depends on its having an inviolate arrangement of all its atoms. Obviously, with millions of atoms composing each particle, this assumption is not subject to positive proof at this time. If, however, this assumption is false the virus "crystals" as known at present obviously do not have a "definite internal structure." Even if the assumption is true, these solid forms are probably not crystals in the strict sense. The infectious ratio does not exceed 0.00001 or so for any of the plant viruses. Unfortunately, we have no estimates of the extent to which the inefficiency of the route of infection contributes to the lowness of this value; but even if 10% of the particles in a preparation were potentially infectious, it would follow from the assumption of the uniqueness of structure required for activity that the remaining 90% would differ structurally from the active 10%. With thousands of degrees of structural freedom of the multitude of atoms in such large particles, the inactive forms would doubtless differ from one another, too. Thus the chances that the six, eight, or twelve nearest neighbors of a given particle in such a "crystal" would be identical with it would be minute indeed, to say nothing of the extension throughout the crystal of regularity on an atomic scale. Pirie (*38*) has discussed some of these points in much more detail than we need go into here.

The point of bringing these matters up for discussion now is that the 10 A resolution of the electron microscope for gold-shadowed preparations finally makes it possible to speak of the properties of *individual* particles. The pictures show that differences exist between particles in a preparation of a "crystalline virus." The question is, are the original particles really different and, if so, which of the forms seen is the infectious one? Perhaps the differences seen are merely artifacts due to the preparation of the specimen, but we have no way of knowing whether this is so. It may be that the artifacts, if such they are, reflect differences between the particles on an atomic scale. A defect such as a broken chemical bond, for example, would, of course, be invisible in the present electron microscope, but it might develop into a visible crack when the defective particle was dried for observation.

## Round Virus Particles

Purified bushy stunt virus preparations are quite homogeneous in the ultracentrifuge, and electron micrographs of these preparations show images averaging about 260 A in diameter (56). X-ray studies showed that these spherical particles group themselves in a body-centered, cubic, close-packed arrangement in dry crystals, and that their diameters are 276 A (7). Williams and Wyckoff's gold-shadowed electron-microscope pictures of these particles indicate that they pack together on the supporting membrane as a collection of peas or billiard balls would pack on a plane, but with some tendency to line up in rows.

The southern bean mosaic virus in purified preparations contains spherical particles about 200 A in diameter, which "are for the most part close packed in an ordered array." Shadowed preparations show two-dimensional close packing and the beginnings of a three-dimensional packing as these two-dimensional arrays are stacked upon one another (40).

The spherical virus particles of tobacco necrosis show somewhat similar close packing, in conventional electron micrographs (55), with about 200 A as the distance between the centers of nearest neighbors.

## The Rod-Shaped Viruses

The protein associated with tobacco mosaic virus is in the form of rods approximately 150 A in diameter, as studied in the electron microscope. The lengths of these rods are uniform in some preparations, being about 3000 A, while other samples contain rods having a wide variety of lengths. The length may also vary with the strain. As seen in Fig. 13 of Zworykin (62; also 55), there is a great tendency for the rods to line up parallel to each other like matches floating on water. This tendency to line up in parallel fashion is also manifest in solutions of this material. The investigator is thus provided with a means of studying oriented preparations of the protein.

By examining the scattering of X-rays traveling parallel to the rods in solution, Bernal and Fankuchen (7) obtained hexagonal patterns indicating that the parallel rods had arranged themselves hexagonally, like matches in a long tube which had been shaken. The spacing between the rods increased from 152 A for the dry "crystal" up to 500 A as the "solid" solution was diluted, suggesting perhaps that some sort of long-range force exists between the particles. Besides these long spacings, these investigators also obtained short spacings, which—being independent of the amount of water between the rods—must, they reasoned, be due to a regularity in structure within the individual rods. From their data Bernal and Fankuchen concluded that the atomic arrangement in the rods must follow the pattern shown in part in Fig. 12. The hexagonal rod is made up of some forty-five similar hexagonal

blocks of atomic groups, 68 A thick, stacked on one another. In turn, each of the hexagonal blocks is divided into three equivalent sectors, as shown. The further subdivisions we need not describe here, but each particle seems to be like a tiny crystal having a close-packed and symmetrically arranged atomic structure.

Two further results throw light on finer details of
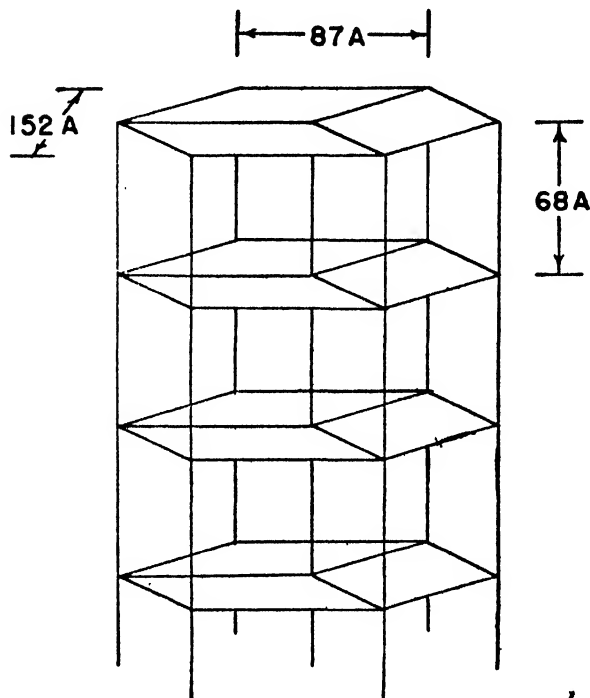


Fig. 12. Diagram of the structure of tobacco mosaic virus protein as deduced from X-ray diffraction studies of oriented preparations (7).

the structure of these particles. Cohen and Stanley (15) report that the nucleic-acid threads extractable from the particles have a "molecular" weight of 300,000 and, like the tobacco mosaic virus rods from which they came, are about 3000 A long. Since 5-6% of the 40,000,000 "molecular" weight particle is nucleic acid, the implication of their work is that each of the tobacco mosaic virus rods has—running lengthwise through it—six, seven, or eight chains of "ribose" nucleic acid.

Another particularly interesting clue to the fine structure of the rods is provided by recent studies of Butenandt et al. (9), who examined the ultraviolet absorption spectrum of flowing solutions of these elongated particles. They found that absorption in bands characteristic of both nucleic acid and tryptophane is greater for light traveling parallel to the direction of flow, and hence parallel to the long axis of the particles, than for light perpendicular to that direction. Now it is well known that the intensity in certain absorption bands of flat aromatic mole-

cules is greater for light traveling perpendicular to the plane of the molecule than for light traveling parallel to it. Butenandt *et al.* deduce from other data that the absorptions they dealt with, not only for nucleic acid but for tryptophane as well, are of this type. The observed dichroism of the particles suggested to them, therefore, that the various aromatic rings of the nucleic acid, and the indole ring of tryptophane also, are largely oriented with *planes perpendicular to the long axis of the particle.*

Other strains of tobacco mosaic virus show X-ray patterns similar to those mentioned; although, as Drs. Stanley and Knight outlined here a few years ago (*57*), certain strains have different chemical compositions. The diameter of the rods of the proteins associated with cucumber viruses 3 and 4 (*55*) are smaller by 4% than the rods of tobacco mosaic virus protein as determined by X-rays (*7*).

## DISCUSSION

Perhaps there are more plant than animal viruses recognized through the symptoms they produce. Those mentioned in the preceding two sections represent a selection which, on the basis of relative stability, availability in quantity, and ease of assay (for many have been neglected because they require an insect vector for transmission), can be handled conveniently in the laboratory. It is thus difficult to say how representative of the group as a whole those studied in detail are.

All the plant viruses so far analyzed are supposed to contain nucleic acid of the pentose type rather than of the desoxypentose type. All the analyzed animal viruses, on the other hand, with the possible exception of that of poliomyelitis, are of the desoxypentose type. Influenza is reported to contain both types (*26*). Whether this chemical dichotomy is real or only apparent must be decided by the results of further research on a greater variety of plant and animal virus diseases.

The matter of selection on the basis of stability raises a further point of interest. It does not seem wholly unreasonable to me, at least, that some of the bodies we speak of today as viruses may at some later date prove to be the stable, condensed, spore-like stages in the life cycles of much larger forms more directly related to the virus inclusion bodies. Thus, associated with the obligate parasite, the virulent *Treponema pallidum,* are seen minute dense forms, which are supposed by some to be capable of developing into the recognizable spirochete. If we were not familiar with closely related free-living forms, it would perhaps be difficult for us to distinguish between the spirochete and an inclusion body or between the tiny granules and viruses.

On the other hand, many of the viruses may be closely related to normal cytoplasmic or nuclear constituents of the hosts (*16*). Indeed the non-Mendelian character-determining entities, such as those discussed in this Symposium—the killer substance (*52*), the pneumococcus transforming principle (*35*), and the enzyme-producing cytogenes (*53*), each of which multiplies within the host—are difficult if not impossible to distinguish from viruses, except that they do not produce an obvious disease in the host. The latter distinction is not great, however, for the killer gene produces the extracellular killer substance, which induces toxic symptoms and death in susceptible protozoa. Some of the plasmagenes that have been studied are particularly useful to the student of filterable forms; for, unlike the viruses, in contributing to the cellular economy they provide a continuous index of their presence and activity within the host cell.

The bacteriophages studied in detail have also been selected from many that are available. The selection is made on the basis of stability, the clearness of the plaques they produce, and the completeness with which they lyse a culture of the host.

It is probable that as more experimentally difficult types are studied many new forms of virus particle will be found to attack plants, animals, and bacteria. But, until we know a great deal more than we do now about the chemical and morphological modes of proliferation of the various forms, any extensive attempt to classify them seems premature.

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# BIOCHEMICAL MUTATIONS IN NEUROSPORA

## DAVID BONNER[1]

Biochemical mutations in Neurospora have been extensively reviewed in the past year (3, 4, 21, 40). Several new biochemical contributions to Neurospora research have been made, however, since these reviews appeared. This paper will therefore consider Neurospora research primarily from the standpoint of biosyntheses of vital substances.

The field of biosynthesis of vital cellular constituents has long held the interest of biochemists. That a cell is able to utilize reactions not at the disposal of the chemist has been appreciated for many years, and few instances have been found where it is possible to describe in detail the chain of reactions involved in the biosynthesis of such vital end products as vitamins and amino acids.

With the use of radioactive and isotopic substances as tracer elements, notable advances have been made in this field. The work of Schoenheimer and his colleagues (32) has gone far towards formulation of the general concepts of cellular syntheses. The use of such markers is handicapped, however, by the difficulty involved in stopping a chain of reactions at each step and so determining the chemical nature of each intermediate in the chain. Biochemical mutations of microorganisms have supplied material in which reaction chains are blocked genetically at successive steps of a sequential series of reactions. Biochemical investigation using such genetic strains, though still in preliminary stages, clearly indicates that these strains will be of great value in elucidating the nature of such biosynthetic intermediates.

Investigations on the genetic control of biochemical reactions in Neurospora and elsewhere (3) have led to the concept that a one-to-one relation exists between chemical reaction and gene. This concept has very direct biochemical implications. Alteration of a single gene will block a single reaction. Mutations of single nonallelic genes which give rise to requirements for the same end product represent genetic blocks of different biosynthetic reactions in the chain of reactions leading to synthesis of this end product. Mutant strains grown upon an exogenous supply of end product will accumulate the intermediate immediately prior to the genetic block, provided this intermediate is not otherwise metabolized. Thus, genotypically different biochemical mutant strains requiring the same end product furnish material in which the consecutive steps of the biosynthesis of this end product can be studied, and provide a possible means of accumulation of intermediates.

## MUTATIONS IN NEUROSPORA

The heterothallic ascomycete *Neurospora crassa* is a favorable organism for this work. Its nutritional requirements are simple, and it can be grown on a chemically defined medium. From a genetic standpoint it is almost ideal. Vegetative cultures are haploid, and problems of dominance are therefore not encountered. Furthermore, recovery of all the products of meiosis permits easy genetic analysis. The linear order of the ascospores also makes it possible to distinguish readily between first- and second-division segregation of alleles.

A method of obtaining mutant strains (6) might be briefly described as follows. Asexual spores of *Neurospora crassa* are irradiated with ultraviolet radiation or with X-rays. These treated spores are then applied to wild-type protoperithecia. Sexual fusion occurs, with the formation of ascospores. Single ascospore isolations are made, and the ascospores are germinated and grown on a medium as nearly complete in growth factors as can conveniently be made. Conidia from this culture are then transferred to a medium of known composition capable of supporting growth of the parent strain. Such a medium for Neurospora culture consists of a carbon source, a nitrogen source, inorganic elements, and one vitamin—biotin. If the strain in question is unable to grow on such a medium, a mutation is assumed. By appropriate testing the additional nutritional requirement is determined.

About 85,000 such single-spore isolations have been made to date, and of these 500 to 600 strains show defective biosynthetic ability compared with the parent strain. Many of the 500 to 600 strains represent recurrences of the same mutations, but there are probably over a hundred mutations involving separate loci (6, 21).

Requirements for each of the B vitamins with the exception of folic acid have been found (6). Nine of the ten amino acids essential for growth of the rat (31) have been found essential for various strains if possible (6). Cystine, proline, and serine requirements have also been found (6). No new strain requiring histidine has yet been isolated in Neurospora. The fact that most of the mutations observed to date involve vitamin and amino-acid synthesis is primarily a reflection of the method used for selecting mutant strains. On the assumption that genes control chemical reactions by controlling the production of specific enzymes (3, 4, 17, 47), appropriate means of detection should yield mutations involving a wide variety of enzymatically catalyzed reactions. Only limited work has been attempted in this direction so far (6, 21).

Attempts have been made recently to induce biochemical mutations in Neurospora by means of chemical treatment. The observation of Auerbach and Robson (1) that mustard gas induces mutations in Drosophilia made it of interest to determine whether this compound would induce biochemical mutations in Neurospora. Horowitz et al. (20) have treated asexual spores of the normal strain with mustard gas. Using techniques described for isolation of biochemical mutants from irradiated material, they have obtained twelve biochemical and

buffered aqueous solution (pH 3.0) gives little killing of the spores, and, while it induces morphological changes, the data shown in Table 1 suggest that it is not highly active in inducing biochemical mutations. In solutions buffered at pH 6.0 the killing rate is very high, and at certain concentrations active in inducing mutations for biochemical characters (Table 1). The difference of effectiveness at the two different pH values may be due to a difference in uptake of the amine. However, in view of the chemistry of nitrogen mustards as reviewed by Gil-

TABLE 1. EFFECT OF DI-$\beta$-CHLOROETHYLMETHYLAMINE ON MUTATIONS IN NEUROSPORA CRASSA[3]

Conidia of 1A suspended in water or buffer (M/10) at pH 6.0, treated 30 minutes with amine hydrochloride, diluted with $H_2O$, centrifuged, resuspended in $H_2O$, and added to protoperithecia of 25a. One ascospore isolated per perithecium, and germinated and tested as usual.

| Series | Amine HCL concentration % | In soln. of | Isolated strains tested | Morphological[1] mutants | | Biochemical[2] mutants | | Killing of Conidia % |
|--------|------|------|------|------|------|------|------|------|
| | | | | No. | % | No. | % | |
| V | 1.0 | $H_2O$ | 700 | 29 | 4.1 | 1 | .14 | — |
| VI | .5 | pH 6.0 buffer | 62 | 1 | 1.6 | 0 | | 98+ |
| | .05 | pH 6.0 buffer | 129 | 5 | 3.87 | 5 | 3.87 | 98 |
| | .005 | pH 6.0 buffer | 175 | 14 | 8.0 | 0 | | 80 |
| | .0005 | pH 6.0 buffer | 270 | 23 | 8.5 | 0 | | 50 |
| VII | 1.0 | pH 6.0 buffer | 57 | 2 | 3.5 | 0 | | — |
| | .1 | pH 6.0 buffer | 119 | 15 | 12.5 | 5 | 4.2 | — |
| | .01 | pH 6.0 buffer | 204 | 7 | 3.4 | 0 | | — |
| | .001 | pH 6.0 buffer | 169 | 8 | 4.7 | 1 | .6 | — |

[1] Include 3 albinos and several "cauliflower."
[2] Lysineless, 4; yeast-extract, 4; $B_6$-less, 2; leucineless, 1; tryptophaneless, 1.
[3] Unpublished work of Dr. E. L. Tatum, Yale University.

seventeen morphological mutant strains from a total of 760 tested. A comparable number of strains tested without treatment gave one doubtful mutant. The mutation rate compares favorably with that obtained by treatment with ultraviolet irradiation. No new specific growth-factor requirements were observed in the biochemical mutants, though some of them may be genotypically different from strains previously obtained.

As pointed out by Gilman and Philips (15), nitrogen mustards have an effect similar to that of sulfur mustards. Tatum (42) has investigated the use of a nitrogen mustard in inducing biochemical mutations in Neurospora, and found it effective. Treatments were made by suspending asexual spores of the normal strain in buffered solutions of di-$\beta$-chloroethylmethylamine. After treatment spores were freed of the amine and crossed with the opposite mating type of the normal strain, and biochemical mutant strains were isolated in the standard manner (6). The amine hydrochloride in un-

man and Philips (15), this difference may be due to the fact that at the lower pH nitrogen mustards do not undergo cyclization and formation of the ethylenimonium compound, which compound is an effective alkylating agent. At the higher pH this cyclization occurs. If formation of the ethylenimonium compound should be found to account for the difference in activity at the different pH values, it would support the view (15) that nitrogen and sulfur mustards induce chromosomal effects through alkylation.

As in the case of mutant strains obtained using sulfur mustard, Tatum has not observed, in the mutant strains whose growth requirements have been determined, any new requirements (42). Genetic investigation, however, may show the presence of new genotypes. The wide variety of mutant strains of Neurospora obtained by use of nitrogen or sulfur mustard suggests that these compounds, like short-wave irradiation, are nonspecific in their chromosomal effects.

## Production of Mutations in Penicillium

A large number of biochemical mutants have been obtained in a second fungus, *Penicillium notatum* (*7*). Since *P. notatum* is an imperfect fungus, no direct proof of the genetic control of biochemical reactions has been obtained. A comparison of the mutants found in Penicillium with those found in Neurospora, however, makes it appear almost certain that vital biochemical reactions in Penicillium are genetically controlled.

While the mutants obtained in Penicillium cannot be used in genetic studies, many of them are interesting from a biochemical standpoint, and are described here because biochemical investigations concerning them will be described later.

About 400 strains having deficiencies in synthetic ability have been isolated from a total of 85,595

TABLE 2. ACCESSORY GROWTH FACTORS REQUIRED BY
BIOCHEMICAL MUTANTS OF PENICILLIUM

| | |
|---|---|
| Biotin | Arginine |
| Choline | Cystine |
| Inositol | Histidine |
| Nicotinic acid | Isoleucine |
| $p$-aminobenzoic acid | Leucine |
| Vitamin $B_1$ | Lysine |
| Vitamin $B_6$ | Methionine |
| Yeast nucleic acid | Phenylalanine |
| | Proline |
| | Tryptophane |

strains (*7*). Table 2 lists the vitamins and amino acids for which mutants of Penicillium are known.

## Biosynthesis of Vitamins

As an example of the value of biochemical mutants for investigation of biosynthetic processes in uncharted fields, the biosynthesis of nicotinic acid will be reviewed in detail.

Five strains of Neurospora have been isolated that require added nicotinic acid, nicotinamide, or related substances for growth (*9*). The activity of nicotinic acid and nicotinamide is identical if correction is made for dissociation of nicotinic acid. Heterocaryon formation, as a test for genetic dissimilarity (*5*), indicates three genetic types. Outcrosses with normal and with each other show segregation, as would be expected of mutations involving three different loci. The fact that mutation of three different genes gives rise in each case to a requirement for nicotinic acid suggests that the synthesis of this compound involves at least three separate reactions. The synthesis might, therefore, be represented as

$$\xrightarrow{\text{gene 1}} A \xrightarrow{\text{gene 2}} B \xrightarrow{\text{gene 3}} C \longrightarrow \text{nicotinic acid.}$$
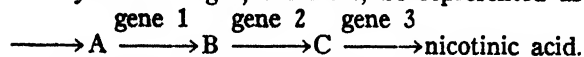
These three strains were tested on a number of compounds of known structure. Such compounds included pyridine, piperidine, piperidine-(3)-carboxylic acid, ornithine, proline, and tryptophane. As no compound of known structure tested proved

active, it was concluded that the blocks in nicotinic-acid synthesis in these three strains involve a set of compounds not ordinarily found in the cupboard of a chemical laboratory.

Since genetic data show that each of these three mutants represents a block in the synthesis of nicotinic acid at a unique point, the strain blocked at step 3 above should accumulate substance C, which both strains 1 and 2 can use for growth, and strain 2 should accumulate B, which strain 1 can use, provided B and C are not otherwise utilized. In the
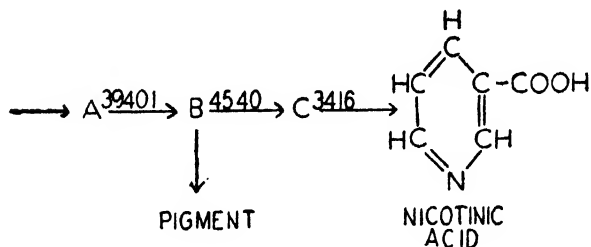


FIG. 1. Nicotinic acid synthesis in Neurospora. Genetic blocks indicated by strain number.

three strains, therefore, the means are available for producing either B or C and assaying for it.

Strain 4540 does, in fact, accumulate in the medium a substance possessing nicotinic-acid activity for strain 39401 (*9*). This substance is inactive for the third genetic type, strain 3416. This suggests that the active substance accumulated by strain 4540 is B, and that the order of the blocked reactions is that shown in Fig. 1. Two active substances have been crystallized from 4540 culture filtrates (*9*). One of these substances is a labile compound with an activity as great as nicotinic acid in promoting growth of strain 39401. The second substance has only a thousandth the activity of nicotinic acid. Both compounds are closely related chemically, and are heterocyclic monocarboxylic acids whose properties suggest that they might be oxy-pyridine carboxylic acids (*9*). The structures of these two compounds have not yet been established.

Strain 4540 has an additional interesting property. When it is grown on nicotinic acid or nicotinamide, a reddish-brown pigment appears in the medium. This pigment production is associated with a fall in precursor activity and represents either abnormal metabolism or spontaneous decomposition of the intermediate. The pure intermediate in aqueous solution decomposes to a colored substance, but whether the two compounds are identical cannot be stated. In any event, pigment production in this mutant is directly related to accumulation of an intermediate of nicotinic-acid synthesis (*9*). Several other cases of pigment formation are known in Neurospora mutants and probably represent abnormal utilization of an accumulated precursor.

The path of choline synthesis in Neurospora has

been determined, using similar techniques. Two choline-requiring strains are known, involving mutations at different loci (*19*). Tests for choline activity of known compounds show that both strains can use dimethylaminoethanol and that neither strain can use ethanolamine, which is known to serve as a choline precursor for rats (*38*). Moreover, strain 47904 cannot use monomethylaminoethanol, while the second strain can (*18, 19*). Horowitz *et al.* (*19*) further observed that strain 47904 accumulates a substance in the medium which has choline activity for strain 34486. Isolation and identification of this substance proved it to be monomethylaminoethanol (*18*). Assuming ethanolamine to be the precursor of monomethylethanolamine and di-

$$H_2N\text{-}CH_2CH_2\text{-}OH \qquad \text{AMINOETHANOL}$$
34486 ----→
$$CH_3\text{-}HN\text{-}CH_2CH_2OH \qquad \text{MONOMETHYLAMINOETHANOL}$$
47904 ----→
$$(CH_3)_2\text{-}N\text{-}CH_2CH_2OH \qquad \text{DIMETHYLAMINOETHANOL}$$
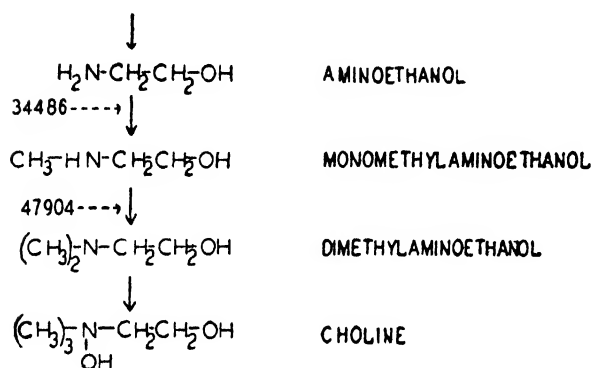$$(CH_3)_3\text{-}N\text{-}CH_2CH_2OH \qquad \text{CHOLINE}$$
$$OH$$

FIG. 2. Choline synthesis in Neurospora. Genetic blocks indicated by strain number.

methylethanolamine to be a precursor of choline, the synthesis of choline in Neurospora can be illustrated as shown in Fig. 2.

Mitchell and Houlahan (*28*) have recently reported studies on a group of mutants requiring adenine for growth. Forty-five independently occurring mutations involving adenine synthesis have been observed, seven of which represent mutations at separate loci. According to previous arguments, this would indicate at least seven separate biosynthetic reactions leading to the production of adenine. All of these strains utilize adenine for growth, all but one utilize hypoxanthine, and none utilizes guanine —suggesting that hypoxanthine serves as a precursor for adenine in Neurospora. Advantage was taken of the pigment production of one mutant in placing the other strains in the sequence. Strain 35203 produces a purple pigment when grown on adenine. This pigment, as in the case of the nicotinic pigment (*9*), is thought to arise by the breakdown or metabolism of an accumulated precursor. As the pigment is related to an adenine precursor, double mutants with strains whose genetic blocks occur prior to that of the strain giving pigment formation would show no pigment production. Strains blocked after this point should yield pigment-producing double mutants. Double mutants of this pigment-

producing strain with other adenine genotypes give rise to pigment production only with the strain unable to convert hypoxanthine to adenine, suggesting the following order of reactions:

genes 3, 4,
5, 6, & 7                gene 2                    gene 1
————→ precursor ————→ hypoxanthine————→ adenine
         ↓
    purple pigment
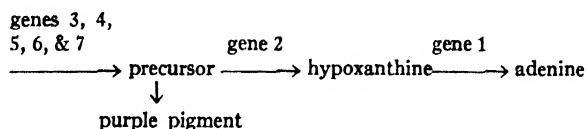
The chemical nature of the precursor is unknown, but genetic studies have done a great deal in formulating a preliminary scheme for the biosynthesis of adenine.

Neurospora is not satisfactory for investigation of biotin synthesis, because the normal strain requires biotin for growth. Biotinless strains of Penicillium, however, have been used by Tatum (*41*), in a manner similar to that discussed earlier for nicotinicless and cholineless strains of Neurospora, to identify one step in biotin synthesis. Penicillium strain 21464 can utilize desthiobiotin as well as biotin. A second Penicillium strain, 62078, requires added biotin, but cannot utilize desthiobiotin. Strain 62078 accumulates a substance in the medium which has biotin activity for strain 21464, and which from a variety of evidence appears to be desthiobiotin. Although desthiobiotin itself has not yet been isolated, it seems highly probable that this substance is a normal precursor of biotin in Penicillium.

Evidence obtained with Neurospora (*44*) is consistent with the view that thiamin synthesis takes place through the production of vitamin thiazole and vitamin pyrimidine and their subsequent coupling, as is found in other organisms (*33*). Two mutants remain unexplained, however, in which a requirement for both vitamin thiazole and vitamin pyrimidine has been found associated with a single gene change. Further study of these mutants is necessary and is of importance to the concept of a one-to-one relation of chemical reaction to gene postulate. A possible explanation of such mutant strains will be discussed later in this paper.

One strain requiring pyridoxin has been reported (*39*). This strain shows marked stimulation of growth after additions of thiamin under conditions of limited pyridoxin. Eight additional strains have been found, all of which show a similar thiamin effect. The relation between pyridoxin and thiamin in these strains is being investigated by Dr. D. L. Harris of our laboratory. He has observed that while pyridoxinless strains accumulate thiamin thiazole in the medium, this is also true of the normal strain. Studies of one pyridoxinless strain, however, show that it accumulates a substance in the medium which possesses thiamin pyrimidine activity (D. L. Harris, unpublished). Though little chemical work has been carried out towards identification of this substance, the preliminary observations suggest that

pyridoxin performs an essential function in thiamin pyrimidine synthesis.

Strains have been described requiring inositol (2), *p*-aminobenzoic acid (43), pyrimidines (24), pantothenic acid (21), and riboflavin (27). In these cases, however, extensive work regarding biosynthesis has not yet been carried out.

### BIOSYNTHESIS OF AMINO ACIDS

In the preceding section several instances of the accumulation of normal intermediates as a result of a genetic block have been described. In these mutants the accumulation of an intermediate in the mycelium and medium has not led to any pronounced growth effect. It is theoretically conceivable, however, that accumulation of a normal intermediate might result in aberrant growth requirements. Intermediates of structurally related end products are probably structurally similar. Either intermediate might act as a competitive inhibitor in the synthesis of the related end products. Normally, intermediates probably do not attain any great concentration, but as shown earlier a genetic block will favor their accumulation. The accumulation of one of two structurally related intermediates might enable the accumulated intermediate to act as a competitive inhibitor of the similar biosynthetic step in the synthesis of the related compound, giving rise to a multiple growth-factor requirement of the mutant strain. Such strains would be expected to have a multiple requirement associated with a single gene change. The mutant strains having a requirement for the vitamin $B_1$ thiazole and pyrimidine analogues, associated with a single gene change, may represent such a case.

There is good evidence that mutant strains requiring isoleucine and valine are mutants of this type.

One mutant strain of Neurospora, 16117, has been described, which has a growth requirement for the two amino acids, isoleucine and valine, associated with a single gene change (10). Five additional strains have been isolated with identical requirements. Genetic and biochemical investigations of these strains indicate that they all represent recurrences of the same mutation present in 16117 (8).

The growth requirement for isoleucine and valine involves a concentration ratio of isoleucine to valine, the optimal ratio being 30% *l*(+)-isoleucine to 70% *l*(−)-valine (10). The reason for this ratio appears to be that in the presence of sufficient isoleucine for growth, increasing concentrations of valine give complete growth inhibition at a concentration about ten times that of isoleucine. The inhibition can be reversed by additional quantities of isoleucine (8). A similar inhibition by isoleucine can be demonstrated at concentrations about ten times the valine concentration. These inhibitions suggest that exogenous supplies of either one of the two amino acids inhibit the utilization of the other. That these inhibitions alone do not account for the double

requirement of strain 16117 can be demonstrated by the fact that strains have been found which will grow on valine alone (8).

Strain 33051 will attain about one-third normal growth on valine alone (8). Additions of isoleucine, however, will bring it to normal (8). Concentrations of isoleucine about ten times that of valine, moreover, will give complete growth inhibition (8). Requirements of this general type are to be expected from the inhibition by exogenous supplies of isoleucine and valine rather than from a competition like that postulated for strain 16117.

Preliminary experiments to locate a point of block in strain 16117 showed the hydroxy-acid analogues to be inactive for growth (10). Crude keto-acid preparations were made by oxidation of the racemic isoleucine and valine, using *d*-amino acid oxidase, and were found inactive when tested in a mixture of the two. The keto-acid preparations were active, however, when tested singly in the presence of the other amino acid (10). As a check on the results obtained using *d*-amino acid oxidase preparations of the keto-acid analogues of these two amino acids, the two keto acids were prepared synthetically (8). Synthetic α-keto isovaleric acid is active for growth of strain 33051, establishing the genetic block in valine synthesis prior to the conversion of the keto acid to the amino acid. Synthetic α-keto isovaleric acid is active for growth of strain 16117 when tested in the presence of isoleucine, establishing the fact that the conversion of the keto acid of valine to valine is not blocked. The synthetic keto-acid analogue of isoleucine, however, is inactive for growth of strain 16117 when tested with valine, in distinction to the activity of preparations of the keto acid made by the use of *d*-amino oxidase. The difference in activity between the two preparations is not due to an inhibiting effect of the synthetic material, since it is active for an isoleucineless strain of *E. coli*. Furthermore, the inactivity of the synthetic keto acid for strain 16117 is not due to the fact that Neurospora cannot convert the keto-acid analogue of isoleucine to the amino acid. Mr. H. J. Teas of our laboratory has investigated a mutant that can grow on either threonine or isoleucine. This strain is able to utilize the synthetic keto-acid analogue of isoleucine for growth. These observations suggest, therefore, that strain 16117 is unable to convert α-keto-β-methyl-*n*-valeric acid to isoleucine. The activity of *d*-amino oxidase keto-acid preparations would then likely be due to the presence of an intermediate between keto acid and amino acid. The keto-acid analogue of isoleucine has further been found to inhibit the conversion of α-keto isovaleric acid to valine in both 33051 and 16117 (8).

The observation that, while the conversion of the keto-acid analogue of valine to valine can occur in 16117, the conversion of the keto-acid analogue of isoleucine to isoleucine is blocked, rules out the possibility of a genetic block of a common step in the

syntheses of isoleucine and valine. The more plausible explanation is that shown in Fig. 3.

This scheme suggests that the single gene change of strain 16117 blocks a single reaction, one of the biosynthetic steps involved in the conversion of the keto-acid analogue of isoleucine to isoleucine. As a result of this genetic block, sufficient keto acid ac-

Over fifty independent recurrences of strains of Penicillium having defective arginine synthesis have been isolated (7). In addition, a number have been isolated which are unable to synthesize proline (7). The arginineless strains may be classified with regard to their growth on arginine, citrulline, ornithine, proline, and glutamic acid as follows:
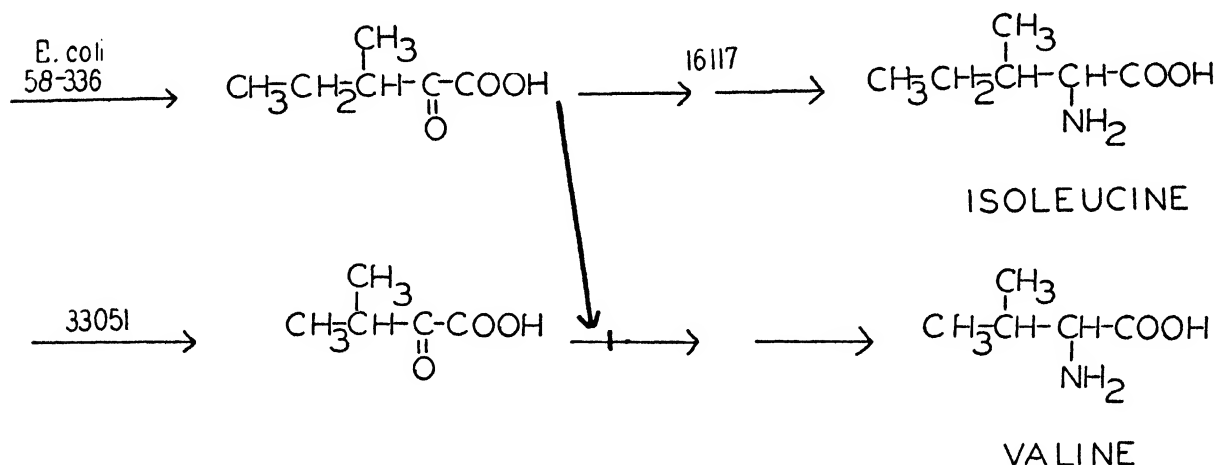


FIG. 3. Synthesis of isoleucine and valine in Neurospora. Genetic blocks indicated by strain number.

cumulates to inhibit the conversion of the keto-acid analogue of valine to valine. Thus a single gene change blocks genetically a biosynthetic reaction characteristic of only one amino acid, but results in an apparent double requirement.

Amination of keto acids is known to occur by transamination (11) and by reaction with ammonia (14). Which method is involved in isoleucine synthesis in Neurospora is not known, and insufficient data have been obtained to permit sound speculation.

The longest chain of genetically controlled reactions in Neurospora to be worked out is the series in the synthesis of arginine. Srb and Horowitz (37) have shown that arginine is synthesized by Neurospora through the chain of reactions known to occur in arginine and urea synthesis in the mammalian liver (23).

The genetic control of each step has been established by the demonstration of a genetic difference between the strains capable of utilizing different intermediates. The genetic data also corroborate the suggestion of an intermediate in the conversion of ornithine to citrulline, since two different genes are found to be essential for this conversion (37). The ornithine cycle in Neurospora may be illustrated as follows:

| Strain number | Growth on | | | | |
|---|---|---|---|---|---|
| | Arginine | Citrulline | Ornithine | Proline | Glutamic acid |
| 3,485 | + | − | − | − | − |
| 6,572 | + | + | (±) | − | − |
| 6,155 | + | + | + | − | − |
| 9,929 | + | + | + | + | − |
| 24,033 | + | + | + | + | + |

Since all five strains can utilize arginine, and but four can in addition utilize citrulline, citrulline must act as a precursor of arginine. Similarly, ornithine is a precursor of citrulline. Strains that can use proline alone are known, which fact would argue that this heterocyclic amino acid is not involved in the primary chain of reactions leading to arginine synthesis. This is in agreement with the suggested cycle of Shemin and Rittenberg (35) for proline synthesis in the rat, though it would appear that proline synthesis does not occur in Penicillium from ornithine, but rather from an intermediate in the conversion of glutamic acid to ornithine. A probable scheme of arginine and proline synthesis in Penicillium is shown in Fig. 4. No proof of the genetic
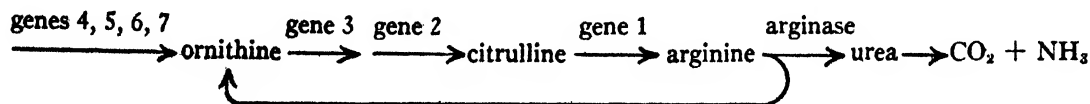
control of each of these reactions has been obtained in Penicillium, yet comparison of these strains with strains of Neurospora leaves little doubt that each of the reactions in the chain shown above is genetically controlled. A similar scheme has been suggested (*36*) to hold for Neurospora, and by use of Neurospora mutants it should be possible to dem-

and serine (*22*) have been reported. Extensive work on the biosynthesis of these compounds has not yet been completed.

### pH- AND TEMPERATURE-SENSITIVE MUTANTS

A number of mutant strains are known which exhibit nutritional deficiencies only under specific en-
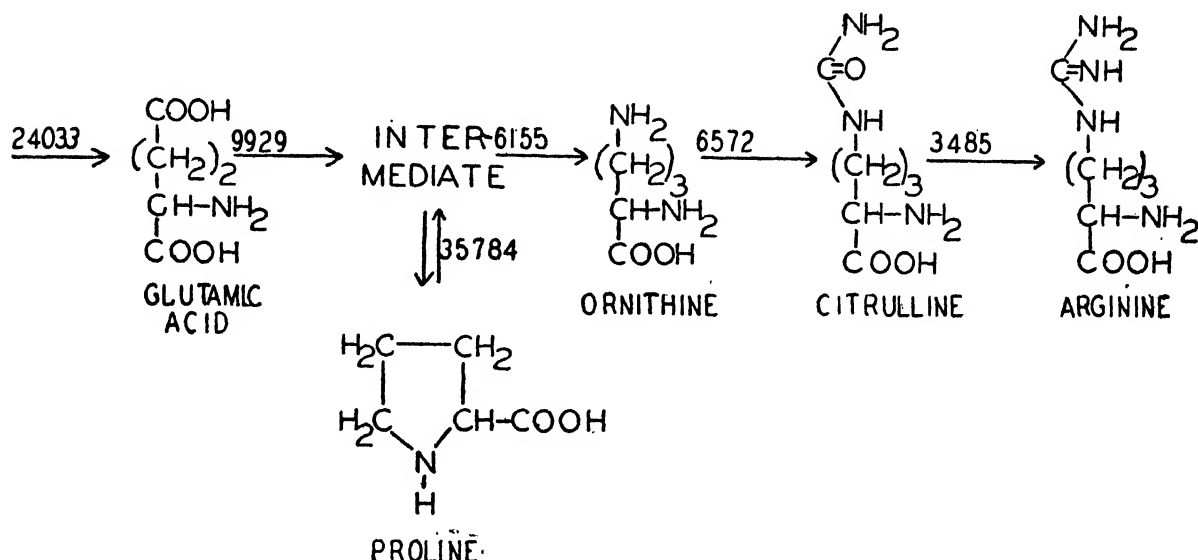
FIG. 4. Synthesis of proline and arginine in Penicillium. Genetic blocks indicated by strain number.

onstrate conclusively the genetic control of each step in the series.

The synthesis of indole from anthranilic acid by Neurospora has been demonstrated (*46*). It has further been shown, following methods outlined earlier in this paper, that anthranilic acid is a normal intermediate in the synthesis of indole. Indole
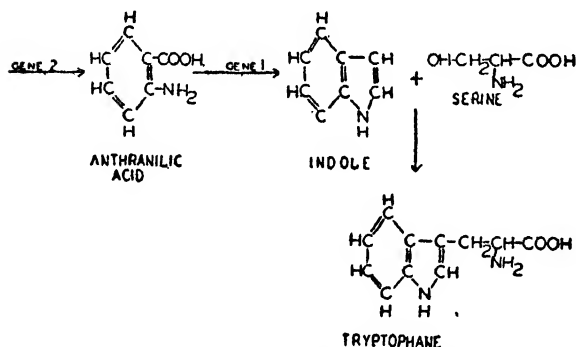
FIG. 5. Tryptophane synthesis in Neurospora. Genetic blocks indicated by strain number.

in turn is then condensed with the amino acid serine, yielding tryptophane directly (*45*). The synthesis of tryptophane by Neurospora is shown in Fig. 5. No mutation blocking the production of tryptophane from indole has yet been observed.

Strains requiring lysine (*12, 13*), leucine (*30*),

vironmental conditions. One of these requires pyridoxin when grown in a medium adjusted to pH 5.8 or less (*39*). This same strain will grow normally without additions of the vitamin in a medium containing ammonia at a pH greater than 5.8. Other pyridoxinless strains are known which are not pH dependent. Mutant strains are also known which require vitamin supplements when cultured at temperatures of 26° or more yet grow normally with no vitamin requirement at lower temperatures (*27, 28*).

Though the nature of gene action is unknown, a logical assumption is that genes exert their control over biochemical reactions through control of the formation of specific enzymes (*3, 4, 17, 47*). Mutations giving pH- and temperature-sensitive strains might then be accounted for by assuming that the gene is altered only to the extent that it controls production of an enzyme with altered physical properties (*e.g.*, heat of inactivation) without affecting its specificity. Such enzymes might theoretically be obtained by breaking certain hydrogen bonds of the protein molecule, giving altered physical properties without radically affecting the spatial configuration, thus maintaining the enzymatic specificity. Biochemical mutations independent of pH and temperature would be similarly accounted for as due to loss of genes, or alterations of genes sufficient to give rise to production of inactive enzymes or enzymes with altered specificities.

An experimental approach to this problem has been attempted by McElroy and Mitchell (*25*). Strain 44206, grown at temperatures above 26° C., requires adenine or adenosine for growth. The requirement for adenine or its riboside cannot be filled by hypoxanthine or inosine. A second nontemperature mutant is known which requires adenine for growth but can utilize hypoxanthine or inosine equally well, suggesting that these compounds are precursors of adenine or adenosine. On the assumption that adenine is normally produced as its riboside from inosine by the enzyme active in the reverse reaction, adenosine deaminase, purified preparations of adenosine deaminase from the mutant strain and from the normal strain were made. Temperature inactivation curves show no appreciable difference between the two. The results of investigations of this one enzyme do not, however, invalidate the argument. The *in vivo* production of adenosine from inosine must be coupled with an energy source, and a genetic block in the energy-yielding reaction might lead to the same growth observations. Furthermore, if the synthesis occurs by way of hypoxanthine, or by way of the nucleotides, different enzyme systems altogether would be involved. Further work is being done on this reaction.

A second explanation that has been advanced to account for such mutations (*4*) is that Neurospora may normally possess two pathways for the synthesis of vital substances, each pathway having a different environmental optimum. Such a scheme would have a definite selective advantage and would be expected to be preserved in the population. Proof in support of such a theory has not yet been obtained.

Still further explanations might be advanced, but critical evaluation must await further work. In any event, these mutations are of utmost interest, and the explanation of their modified activity may well contribute generously to theories of the nature of gene action.

### SUMMARY

Studies on biochemical mutations in Neurospora have led to unequivocal proof of the genetic control of vital biochemical reactions in this organism. From investigation in other organisms (see review by Beadle, *3*) it seems logical to assume that such a genetic control is not limited to Neurospora but rather exists in all living cells. Studies of genetically blocked reactions have further substantiated the postulate that there exists a one-to-one relation between chemical reaction and gene. No case has been observed in Neurospora which contradicts this postulate.

A body of work has been accumulated strongly suggesting that genes exert their control over biochemical reactions by directly or indirectly controlling the production of specific enzymes (*3, 4, 17, 47*). The direct demonstration that mutations in Neurospora involving biochemical reactions are associated with lack of production of a specific enzyme has not been accomplished. The synthetic reactions studied to date are notoriously difficult to work with, since they ordinarily require energy. Indirect evidence on this point has, however, been obtained.

If genes control production of enzymes, a genetic block in a reaction chain would be expected to result in accumulation of the substrate immediately prior to the point of block. Accumulation of such intermediates would, therefore, constitute indirect evidence that genes exert their control over biochemical reactions by conditioning the production of specific enzymes. Several cases of intermediate accumulation have been noted in Neurospora.

There is quite general agreement at present that genes contain nucleoprotein as an essential component of their structure (*16, 26, 29, 34*). One should expect, therefore, that genes, like other proteins, have specific configurations, the configuration of a single gene being characteristic of itself alone. These various considerations suggest the view that the gene controls biochemical reactions by imposing, directly or indirectly, a specific configuration on the enzymes essential for the specific reactions.

Studies on the biosynthesis of vital substances, using mutant strains of Neurospora, have strengthened the conviction that vital substances are synthesized through a chain of reactions characteristic of single end products. The use of these mutant strains has made possible, in several instances, the prediction of the minimum number of reactions that may be expected in the synthesis of a specific end product, as well as the elucidation of the pathway used by Neurospora in the synthesis of certain vitamins and amino acids.

The chemical nature of intermediates in the biosynthesis of such substances as vitamins is poorly understood. The substances themselves may often be chemically rare or unknown, and their recognition is likely to come only by isolation and identification from natural products. Genetic blocking of biochemical reactions, as illustrated in mutant strains of Neurospora, offers a powerful tool for investigations of this kind.

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## DISCUSSION

DELBRÜCK: It has been pointed out by the speaker that the evidence accumulated in the Neurospora work is compatible with the assumption that there exists a one-to-one correlation between genes and the various species of enzymes found in the cell; and the hypothesis is advanced that quite generally the function of genes consists in imparting the final specificity to enzymes.

The question arises whether the evidence obtained actually supports the thesis, beyond the mere fact that it is compatible with it. The possibility exists, and should be discussed, that the experimen-

tal approach applied in the Neurospora work is such that incompatibilities would be unlikely to arise even if the thesis were not true at all.

The thesis might be untrue in two ways:

(1) In general, one gene may be responsible for a variety of enzymes of different final specificity, in such a fashion that many reaction steps are blocked if this particular gene is put out of action, and that the deficiency cannot be made up by the addition of one essential factor supplied from the outside.

It is clear that the method of analysis employed in Neurospora work is very unlikely to pick up mutations having such effects. This is so because if a mutation has the effect of blocking directly a number of steps then it is very likely that one or the other of the reaction products thus eliminated from the internal metabolism of the cell is of the non-diffusible kind and that the mutation is therefore lethal under any conditions. In other words, the method of detecting mutations employed in Neurospora work seems to be exceedingly selective for mutations with very restricted effects of a special kind.

(2) In general, several different genes may be responsible for the final specificity of any given species of enzyme, in the sense that if any one of these genes is put out of action, this particular enzyme, and only this particular enzyme, will be missing, as a primary effect.

If this were so, one would expect that genes affecting the same step of a synthetic chain, should, in general, not be allelic. Independent mutants, apparently blocked at the same step in the synthesis of the same end product, have been found. In some of these cases, heterocaryons are found to have wild-type characteristics, thus showing that actually two different steps are involved. In these cases, the genes involved have always been found to be non-allelic. In other cases, heterocaryons are unsuccessful. In such cases, presumably, the same step is blocked. Is it true that in such cases the loci are always allelic? If a case were found where the two loci were not allelic, the thesis would be contradicted.

To sum up: in order to make a fair appraisal of the present status of the thesis of a one-to-one correlation between genes and species of enzymes, it is necessary to begin with a discussion of methods by which the thesis could be *dis*proved. If such methods are not readily available, then the mass of "compatible" evidence carries no weight whatsoever in supporting the thesis.

BONNER: To answer your second point first, no carefully studied case has revealed independent mutations affecting the same step of a reaction sequence in which two nonallelic mutations control in identical manner the same chemical step. This, of course, does not rule out the possibility.

Investigations of biochemical mutants of Neurospora have led to the observation that loss of the ability to carry out a specific reaction is associated with the loss or alteration of a single gene. This has been well substantiated in about fourteen separate cases. Although it is not proved, there is good reason to assume that this loss of ability to carry out a specific reaction is associated with the loss of a specific enzyme. The concept has therefore evolved from the cases studied that single genes control the production of single enzymes. This does not imply that a single gene controls the entire production of a single enzyme. Probably many genes are necessary in the production of the requisite polypeptide structure. The specificity of a single enzyme, however, in these cases does appear to be controlled by a single gene.

To come more specifically to your question. The reactions studied in Neurospora are admittedly of essentially similar type; i.e., reactions involved in the synthesis of vitamins and amino acids. Deductions as to the nature of the genetic control of the biosynthesis of more complex compounds, such as polypeptides and polysaccharides, may not be valid on the basis of work done with Neurospora. It should be pointed out, however, that evidence substantiating the one-one concept is not limited to that concerning the synthesis of simple compounds. The work of Lindegren and Spiegelman on the genetic control of glycolytic enzymes in yeast is in strong agreement with the one-one concept.

The one-one concept is not intended as a law. Rather it is intended as a useful working hypothesis, and as such it serves an important function. Exceptions to the one-one concept may well exist. They have not been found in Neurospora, but as you point out, since selection of mutant types is limited in Neurospora to what can be added through the cell wall, selection has yielded mutations of only one general type. The work on yeast would tend, however, to weaken the argument that the observed one-one relation is due to the restricted method of selection of mutant types in Neurospora.

Although your appraisal of the present status of the one-one concept is accurate, I disagree with your summary. As seen from the discussion of the participants regarding the "critical experiment" by which the thesis could be disproved, an experiment is difficult to devise which would yield *no* alternative explanation. This does not, however, in my opinion invalidate the general thesis as a useful, simple working hypothesis. Since the data now available are accounted for on a one-one basis, it would be of little value to devise a more complex thesis of the relation of genes to enzymes.

WHITE: Did you observe any series of multiple alleles, and would you say something about the "one-to-one" theory in relation to multiple allelism?

BONNER: Experimental results obtained in two sets of Neurospora mutants suggest the possibility of multiple allelism. Both instances are found in pH- or temperature-sensitive mutants. As I mentioned earlier, one strain is known to require pyridoxin, but to be sensitive to pH; i.e., it requires

pyridoxin at pH 5.8 or less but is independent of this vitamin at pH values above 5.8. A second strain has been isolated which requires pyridoxin but is pH-independent. Genetic analyses of these strains suggest that the pH-sensitive and the pH-independent strains represent allelic mutations. Similarly, Dr. Mitchell and Mrs. Houlahan have observed apparent allelism in two adenine-requiring strains, one of which is temperature-sensitive and the other temperature-independent.

As I discussed earlier, one possible interpretation of the pH- and temperature-sensitive mutations is that the gene is altered only to the extent that it controls the production of an enzyme with altered physical properties but with similar specificity. The allelic pH- or temperature-independent mutation might then represent alteration of the same gene to the extent that it controls the production of an inactive enzyme. Multiple allelism in these two cases might therefore be considered as alterations of a single gene to different extents, one alteration resulting in enzymes with similar specificity but altered physical properties, and the other in inactive enzymes. These cases are not in conflict with the one-one concept.

ALTENBURG: The Neurospora evidence does not rule out the possibility that one enzyme, like any other trait, is controlled by the interaction of many genes, or that one gene may influence many enzymes. But the evidence does strongly indicate that the *specific* character of an enzyme is ultimately determined by a single gene. For, if this were not true, it would be difficult to see why the *same* locus should always be involved when several recurrent mutations (of independent origin) prevent a given step in a chemical synthesis, particularly when it is remembered that as many as fifteen or more such steps have been worked out.

# THE INHERITANCE OF COLOR IN BACTERIA, WITH SPECIAL REFERENCE TO SERRATIA MARCESCENS

MARY I. BUNTING

It is a very great privilege for me to take part in this Symposium and one for which I am extremely grateful, particularly because my own studies in the field of bacterial genetics were interrupted six years ago. I think it wise to confess here and now that any original work that I may have to report hails from that prehistoric era before Beadle and Tatum (2) isolated their first mutant strain of Neurospora. The tremendous advances which have been made since 1940, and which are so very evident to me, are an impressive token of the fruitfulness of the researches of the members of this Symposium. Certainly the bacteriologist has been given a new viewpoint; perhaps even old data will be of interest when seen with new perspectives.

Although in the past 60 years many bacteriologists have seen theoretical advantages in the use of pigmented species for the study of bacterial heredity, it must be admitted that our knowledge of the inheritance of color remains rudimentary. The reasons for this are perhaps not far to seek. The mutation from red-eye to white-eye in the fruit fly was conspicuous, and by counting the numbers of eyes of each color in the progeny from a mating it was relatively easy for the geneticist to discover and demonstrate the laws of segregation and dominance that govern the sexual transmission of the character. No such regularity was revealed to the bacteriologist, for reasons that have been generally appreciated only recently. His observations were made on cultures, or at best colonies—not individuals—and the variations he saw often appeared continuous rather than discrete. Moreover, there was, and is, no evidence that the organisms studied reproduce sexually; so that in those rare instances in which a statistical approach was attempted (16) there was no *a priori* reason to expect a Mendelian ratio. Furthermore, variations akin to mutations and back-mutations often occurred with confusing frequency, and the display of color was constantly being modified by changes in the pH or O-R potential of the medium. The pigments of bacteria are often as elaborate as those of higher organisms (13, 15, 22, 23, 27) and their syntheses very probably depend on as many genes. Thus, as genetic material, the pigmented bacteria first revealed the faults of their virtues; thanks to their rapid rates of multiplication and the tremendous populations that could be surveyed in a limited time, a baffling array of variants swiftly confounded the observer. Students of color inheritance in the fruit fly, the dahlia, and the guinea pig are faced with an equally complex picture but they have been able to discover

it in a more leisurely fashion. Small wonder that the bacteriologist should have called things by names of his own invention.

There are of course a great many different pigmented forms of bacteria, ranging from the purple sulfur group in which the pigment functions not unlike chlorophyll (24) to the familiar ever-sporting micrococci, which toss off colorless variants with such ease that one doubts the pigment can have any importance in their economy, and wonders that they survive in the pigmented form at all. Although variants are the rule in some species, there are others in which pigmentation is remarkably constant. Thus, in a study of the carotenoids of *Corynebacterium carotenum* and *Mycobacterium phlei* (14), no single color variant was noted over a three-year period. By modifying the cultural conditions it was possible to suppress the appearance of the pigment, but in every case, when conditions were favorable, the synthesis of abundant color was again resumed. Even exposures to X-rays have failed to produce a colorless variant of *Acetobacter melanogenum* (10), and van Niel (25) reports that he has never seen a color variant among the purple sulfur bacteria. Obviously we know little about the mechanism of inheritance of pigment in these stable species.

Without any question the most detailed studies of color inheritance in bacteria have been made with the miracle bacillus, *Serratia marcescens* (11, 12, 16, 17, 19, 20, 21, and many a buried dissertation). Perhaps there is no better meeting ground for the bacteriologist and the geneticist, for here is a bacterium that no bacteriologist can classify away and here is such striking color variation that no good geneticist can ignore it.

No attempt will be made to give a complete review of the literature on color variation in Serratia, but a few references will be cited to illustrate the types of study that have been made. As early as 1888, Wasserzug (26) published a remarkable paper in which he reported that when a perfectly red colony was streaked on a gelatin medium not all of the cells gave red daughter colonies. He found that the number of colorless colonies was particularly large if the gelatin was inoculated from liquid cultures. It was possible by continued replating to obtain colorless colonies that did not revert, although reversals commonly occurred with his first isolates. There also were variations in morphology, which were particularly evident in old cultures. He claimed to have made similar observations on other pigmented species, but details were

not given. His results with Serratia have been confirmed many times.

Eisenberg in 1914 (*9*), to quote another publication, obtained 22 varieties from 7 strains of Serratia. He classified his color variants as dark-red, red, orange-red, rose, light-rose, white-pink, and white. He found that different strains had quite different tendencies to mutate, that mutations were commonly obtained in old cultures, and that the rate of mutation was increased by the presence of dyes. Similarly, in 1932, Daddi (*8*) isolated 8 different color types from a three-day-old culture: red, hyaline-red, mucous-red, gilt, rose, pale-rose, white, and mucous-white. His hyaline-red and gilt strains were "entirely stable"; the other types were more or less unstable.

There are numerous other examples of similar methods of study leading to similar confusion. In color differentiation was better under acid conditions, and we found that when the 3-day-old peptone plates were flooded with dilute acid the orange tone was lost and the colonies varied abruptly from dark-red to bright-pink to pale-pink to white. We therefore made up a synthetic medium that was buffered heavily with dipotassium phosphate, and on this we obtained well-differentiated colonies. The medium contained per liter of water: ammonium citrate, 5.0 g.; glycerol, 5.0 g.; dipotassium phosphate, 10.0 g.; magnesium sulfate, 0.5 g.; sodium chloride, 0.5 g.; ferric ammonium citrate, 0.05 g.; agar, 15.0 g.

The stability of the different color types was studied by picking 3-day-old colonies to water blanks, shaking and diluting the suspensions, and replating on the synthetic medium. The analysis of the colonies so obtained is shown in Table 1.

TABLE 1. ANALYSIS OF COLONIES OBTAINED BY PLATING S. MARCESCENS 274 ON BUFFERED AMMONIUM CITRATE GLYCEROL AGAR

| Color of Parent Colony | Number of Colonies Selected | Average Number of Daughter Colonies Counted | Per Cent of Daughter Colonies Which Were | | | | |
|---|---|---|---|---|---|---|---|
| | | | Dark-red | Light-red | Bright-pink | Pale-pink | White |
| Dark-red | 10 | 497 | 98±1 | + | 2±1 | 0 | 0 |
| Light-red | 5 | 382 | 60±5 | + | 40±5 | 0 | 0 |
| Bright-pink | 10 | 508 | 41±2 | + | 58±2 | + | 0 |
| Pale-pink-1 | 7 | 637 | 19±4 | + | 53±3 | 28±4 | 0 |
| Pale-pink-2 | 3 | 578 | 14±3 | + | 58±8 | | 27±11 |
| White | 5 | 512 | 5±2 | + | 31±3 | | 63±6 |

+ indicates less than 1%.

1937 Reed (*17*) picked up the pearl and called attention to the fact that in Serratia color varied independently of colony type and that both of these were independent of the presence or absence of capsules. Reimann (*18*) made similar observations with another chromogenic organism, *Micrococcus tetragenes*. This was a definite advance, but quantitative studies were still conspicuous by their absence. Little advantage was taken of the fact that the variants were often easy to detect and therefore easy to count, and that by counting them under proper conditions one might obtain information about rates of variation and factors that could affect rates of variation. It seemed to us that such knowledge might throw light on the mechanisms involved in the production of the variants.

We therefore set out to count. The American Type Culture Collection strain #274 of *Serratia marcescens* was selected for intensive investigation (*3*). This strain was typical of the species with respect to morphology and cultural characteristics. When the stock culture was plated on an ordinary beef-extract-peptone-agar medium, a multitude of variants was produced; but unfortunately these varied continuously from deep-orange-red through paler shades to white. Reed (*17*) had stated that

The dark-red colonies were found to be the most stable, but even these invariably contained cells that gave rise to colonies of one of the other color types. Approximately 98% of the viable cells from 3-day-old dark-red colonies gave dark-red daughter colonies, while 2% of the cells gave colonies of the bright-pink type. No pale-pink or white colonies were found on these plates.

The bright-pink colonies contained about 58% of cells that gave bright-pink colonies on replating, and 41% of variants of the dark-red type. Occasional pale-pink colonies were evident on these plates. From this it was judged that the bright-pink type was quite unstable and frequently reverted to dark-red. It also produced a relatively small number of variants of a third or pale-pink type.

On the original plates, and occasionally on plates from later analyses of the dark-red and bright-pink colonies, there were a few colonies of an intermediate light-red color. When these were replated, as may be seen from the table, they were found to be made up primarily of cells of the dark-red and bright-pink types rather than of cells of a specific light-red type. These colonies were judged to be fortuitous mixtures of dark-red and bright-pink

cells; either the colonies had arisen from small mixed clumps or a variant had been produced very early in the development of the colony.

Ten pale-pink colonies were picked from the original plates. Analyses revealed that they belonged to two different groups. Seven, designated pale-pink-1, regularly gave daughter colonies of the pale-pink, bright-pink, and dark-red types. It may be mentioned here that later analysis of the pale-pink colonies that had come as new variants from the bright-pink cells described in the previous paragraph proved that these all belonged to the pale-pink-1 group. The other three pale-pinks from the original plating, designated pale-pink-2, were made up of cells that gave white as well as pink and dark-red daughter colonies.

The white colonies from the plating of the original stock strain gave 68% white daughter colonies,

93% and 99% of cells of the dark-red type, and the remainder were preponderantly bright-pink. The appearance of a new white-type colony from any of the more highly pigmented colonies was relatively rare.

Thus it became evident that, although *Serratia marcescens* #274 was highly unstable with respect to color, the rates at which the different variations occurred appeared to be remarkably constant. Selective replatings over a period of six months did not modify the stability of any of the variant types. This was interesting in view of the fact that a few stable white and pale-pink strains have been encountered in the course of aging experiments to be described later.

All of the evidence from the plating experiments indicated that variants were produced at constant rates. In order to get some quantitative estimate

TABLE 2. RATE OF INCREASE IN THE PER CENT OF DARK-RED CELLS IN FAST-GROWING CULTURES, INOCULATED WITH MIXTURES OF DARK-RED AND BRIGHT-PINK-TYPE CELLS

| | Series Number | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| | Color of colony used as inoculum | | | | | |
| | Dark-red | Dark-red | Light-red | Light-red | Bright-pink | Bright-pink |
| Generation time in minutes: | | | | | | |
| First 2 days | 65 | 65 | 65 | 65 | 65 | 62 |
| Entire 12 days | 64 | 64 | 65 | 65 | 64 | 64 |
| Per cent of dark-red colony forming cells in: | | | | | | |
| Original inoculum | 93 | 93 | 66 | 66 | 23 | 23 |
| 2-day cultures | 96 | 95 | 79 | 80 | 45 | 44 |
| 4-day cultures | 96 | 94 | 86 | 83 | 62 | 61 |
| 7-day cultures | 98 | 98 | 88 | 89 | 78 | 74 |
| 10-day cultures | 96 | 98 | 91 | 88 | 84 | 85 |
| 12-day cultures | 97 | 98 | 92 | 94 | 87 | 88 |

31% pink, and 5% dark-red. The pale-pinks from these plates were principally of the second type described above, and it must be admitted that in the presence of variants of the white type the clear distinction between bright-pink and pale-pink colonies was often obscured. It does seem clear that colonies of the pale-pink-2 type are made up of variants that have reverted from the white type.

From all of these results it was apparent that colonies of four of the types—i.e., the dark-red, bright-pink, pale-pink-1, and the white—contained an appreciable number of cells capable of reproducing the parent type, but that none of these was stable and that variations could proceed in both directions. In order to test these observations further, colonies of each of these four types were selected from plates of the different series and were replated on synthetic agar. It was found that the source had no effect on the composition of the colonies except in the case of the pale-pinks, which has already been discussed. Thus, a three-day-old dark-red colony from any source contained between

of this variation it was essential to have the cells multiplying at constant rates—i.e., growing under logarithmic conditions. This was achieved in a very simple manner (4). The ammonium-citrate synthetic medium was made up without agar, and was tubed in 10-ml. amounts in uniform 1-inch test tubes. These were routinely inoculated with approximately 10,000 cells per ml. and were incubated at 30° C. until their turbidities indicated about 10 million cells per ml. Transfers were then made to fresh tubes, using 1 ml. of a 1:100 dilution of the turbid culture. By transferring in this manner it was possible to keep the cultures growing logarithmically over long periods of time; and from the records of the numbers of transfers the rates of cell multiplication were calculated. The relative numbers of cells of each color type were found by plating on the synthetic agar medium and counting the surface colonies of each color after three days' incubation at 30° C.

Typical results are presented in Table 2. Two series were inoculated from a 3-day-old dark-red

colony, two from a 3-day-old light-red colony, and two from a 2-day-old bright-pink colony which contained a relatively low percentage of dark-red-type cells but no pale-pink or white variants. It will be seen that the rates of growth were the same in each case but that the percentage of dark-red cells increased. Since there was no significant difference between the rates of cell multiplication of the dark-red and bright-pink types, it was concluded that the observed shifts in the compositions of the cultures must be due to differences in rates of variation. The populations appeared to come to equilibrium with about 97% dark-reds and 3% bright-pinks. This was confirmed by later experiments in which the cultures were maintained in the logarithmic growth phase for 30 days. Thus it appeared that under these conditions of growth 97 dark-red cells produced one variant pink cell in the same length of time that 3 bright-pink cells gave rise to one variant dark-red. Such a population may be described by the equation:

$$(1) \qquad R\infty/P\infty = K = Pr/Rp$$

where $R\infty$ and $P\infty$ are the %s of dark-red and bright-pink colony-forming cells at equilibrium, K is a constant, and Pr and Rp are the %s of bright-pink and dark-red cells giving variants of the opposite type in a given time. Substituting $R\infty = 97$ and $P\infty = 3$, one gets $Pr = 32.3 \, Rp$.

Furthermore, the relative number of cells of each color type in similar cultures not at equilibrium is described by the equation:

$$(2) \qquad Rt = Ro \, (1\text{-}Rp) - Po \, (Pr)$$

where Ro and Po are the %s of dark-red and bright-pink cells in the culture at a given moment, Rt is the % of dark-reds after an interval t, and Rp and Pr are again the %s of red and pink cells that form variants of the opposite type in the interval t. Using this equation and substituting values from Table 2 (series 5, 2 to 4 days), Rp was calculated to be 0.0099, indicating that about 1 in 10,000 dark-red cells produced a bright-pink variant in the two-day interval, whereas 32 in 10,000 bright-pink cells produced dark-red variants in the same time interval.

Experiments have also been performed in which cells from pale-pink-1 and white colonies were maintained in the logarithmic growth phase. The results are indicated in Figs. 1 and 2. The series shown in the first graph was inoculated with a pale-pink colony. The numbers of pale-pink cells fell steadily, until equilibrium was reached after approximately two weeks with the usual 97% dark-reds and 3% bright-pinks. The second graph shows a series inoculated from a white colony. In this case there was an unmistakable rise in the percentage of bright-pinks during the early stages, when the numbers of white and very-pale-pinks were decreasing most rapidly. Evidently these were converted into bright-pinks more rapidly than the latter gave rise to dark-reds. Again the records of transfers gave no evidence of differences in growth

rates, so that all of the changes in the fast-growing populations must be ascribed to differences in the stabilities of the different color types. All of these results are entirely consistent with the original plating experiment. They indicate step-wise variation at a rapid rate in two directions.
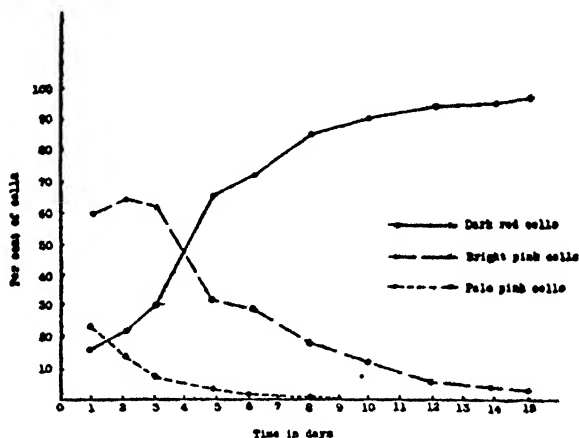


FIG. 1. The distribution of color types in a fast-growing culture inoculated from a pale-pink colony.

Very different were the results obtained as soon as cultures were allowed to age (6). Large flasks of ammonium-citrate-glycerol broth were inoculated with cells from colonies of each of the four major color types. The flasks were incubated at 30° C. and at intervals they were shaken vigorously and
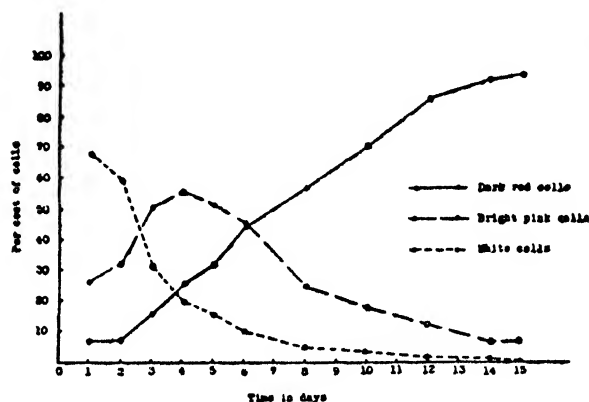


FIG. 2. The distribution of color types in a fast-growing culture inoculated from a white colony.

sampled by the usual methods of plate analysis. The results are shown in Fig. 3. There was an early stage in which the percentage of dark-red-type cells increased as it did in the previous experiments. This was followed by a second phase in which the trend was entirely reversed; and, if one followed the cultures long enough, there was a terminal phase in which the dark-reds once more gained the ascendancy. One suspected that during this third phase

the composition of the cultures was again controlled by the relatively greater stability of the dark-red-type cells; and one's interest was focused on the second phase, in which other factors obviously affected the situation.

Analysis of the second phase showed, as may be



FIG. 3. The percentage of dark-red cells in aging broth cultures inoculated from colonies of four color types.

seen from Fig. 4, that the absolute number of pink variants continued to rise for some time after the total viable count reached a maximum. Either these cells were capable of multiplying under conditions that were not favorable to the dark-reds, or the conditions were such that the "balance of variation" was from red to pink.

One might suspect that the second phase was initiated upon the depletion of some minor element such as iron or magnesium, but there was no evidence for this from experiments in which the various ingredients of the medium were incorporated in reduced or increased amounts (5). Furthermore, it was observed that when the source of carbohydrate

was increased it was possible to postpone the onset of the second phase. When this was done there was a very heavy growth of cells, and the drop in the percentage of dark-reds, although delayed, was especially pronounced, as may be seen by comparing the controls shown in Table 3. In general it
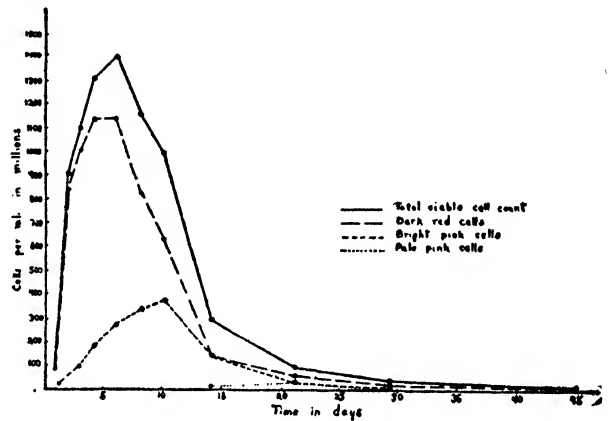


FIG. 4. The distribution of color types in an aging broth culture inoculated with cells from a dark-red colony.

was observed that the greater the growth, the greater was the magnitude of the change in the second phase.

These results suggested that some product of growth, either a metabolic by-product or some substance released from autolysing cells, might be responsible for the characteristic drop in the percentage of dark-reds during the second phase in aging cultures. This was verified when it was found that either heat-killed cultures or their filtrates, when added to new cultures, served to shorten the first phase and accentuate the second. Moreover, the autolysate of washed cells that had been grown on an agar medium was equally effective, as may be seen from Table 3. The effect was doubtful in distilled-water suspension of cells, but quite pro-

TABLE 3. THE EFFECT OF AUTOLYSATE ON THE PER CENT OF DARK-RED CELLS IN BROTH CULTURES INOCULATED FROM A DARK-RED COLONY OF THE STABLE SUBSTRAIN

| Medium | Autolysate per Flask | Per Cent of Dark-red Cells After | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2 days | 4 days | 7 days | 10 days | 13 days | 17 days | 25 days |
| Distilled water | ml. 0 | 96 | 94 | 96 | 97 | 97 | | |
| | 10 | 96 | 96 | 95 | 97 | 99 | | |
| | 20 | 95 | 94 | 94 | 94 | 98 | | |
| 1% glycerol broth | 0 | 98 | 97 | 92 | 88 | 97 | 97 | |
| | 10 | 95 | 64 | 84 | 83 | 92 | 97 | |
| | 20 | 94 | 63 | 68 | 61 | 92 | 98 | |
| 5% glycerol broth | 0 | 95 | 95 | 94 | 97 | 87 | 65 | 50 |
| | 10 | 96 | 83 | 83 | 68 | 67 | 41 | 42 |
| | 20 | 97 | 87 | 73 | 72 | 56 | 36 | 38 |

nounced in the synthetic medium with 1% glycerol. Very low values were obtained when autolysate was added to cultures grown in 5%-glycerol broth.

It was thought that more potent autolysates might be made with the aid of a suitable detergent; but before such a preparation was attempted the effect of sodium lauryl sulfate was tested on the aging cultures. The results were very striking and, to us, very surprising; they are shown in Fig. 5. With 0.05% of the detergent the dark-red cells fell to less than 20% of the population, a figure considerably lower than any that we had previously encountered. The mechanism through which the detergent acted has not yet been elucidated, but it was found that when sodium lauryl sulfate was added to rapidly growing cultures it had little effect on the growth and none whatsoever on the proportions
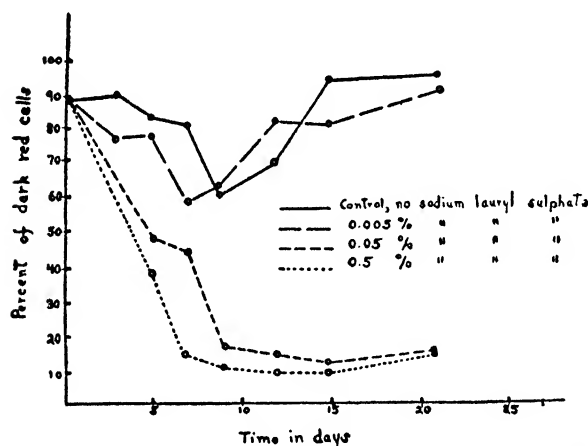


FIG. 5. The effect of sodium lauryl sulfate on the percentage of dark-red cells in aging broth cultures inoculated from a dark-red colony.

of cells of each color type. The cultures came to equilibrium, with 97% dark-reds and 3% bright-pinks, at the same rate as did controls without the detergent. The growth of the cells was affected only slightly, as had previously been found by Cowles (7). This was additional evidence that the agent acted by virtue of its ability to autolyse cells; for it is well known (1) that young Gram-negative cells are quite resistant to this action, whereas cells that have aged slightly are sensitive. This still does not tell us whether the detergents (Drene, Aerosol, saponin, and others were also effective) acted differentially because the dark-red cells were more susceptible to autolysis, because autolytic materials were released that promoted the growth of the bright-pinks, or because materials were released that induced the more highly pigmented types to produce paler variants.

A number of general observations have bearing on these questions. First, it was always noted that in aging cultures the second phase was not initiated until the flasks gave a good foam test on shaking.

In other words, cell autolysis was well under way before the dark-reds decreased. Second, it was not until about the same time that the sediment of cells, even in flasks inoculated from dark-red colonies, contained much pigment; during active growth there was very little accumulation of pigment in the cells. This was borne out by the commonplace observation that small, well-separated, young Serratia colonies were not pigmented, whereas older ones of the same size, whose growth had been checked by crowding, were brightly colored. There was a third finding that may also be worth mentioning; namely, that on a given plate the pink colonies were uniformly larger than the dark-red ones. This might be interpreted as additional evidence of a factor that selectively checked the growth of the dark-reds. Admittedly we have no crucial evidence on the mechanism that accounts for the relative increase of the less highly pigmented cells during the period immediately following active growth in aging cultures.

In concluding this description of the experimental work with Serratia marcescens #274, a few points may be emphasized. Most important was the fact that throughout the aging experiments, when colonies were picked from plates for reanalysis they almost always gave distributions of color types comparable to those obtained from the colonies originally studied. The exceptions were rare completely stable white or pale-pink strains. Also, we have repeatedly confirmed Reed's observations concerning the independence of the color variations; rough and mucoid variants were noted from time to time in any or all of the color types.

It is also important to emphasize the fact that other strains of Serratia behave quite differently from #274. Of 25 cultures that we studied in some detail, 10 resembled #274 in giving bright-pink and pale-pink variants and in the tendency of these variants to revert to the dark-red type. There was a second group, however, that threw only pure-white variants, and these were remarkably stable. A third group of lavender-pink strains gave variants of a lighter hue and infrequently a few dark-reds.

Thus it is seen that in Serratia marcescens #274 there are striking examples of heritable color variation (or mutation, if one uses the word synonymously). The specific variations are abrupt, easily recognizable, reversible; they occur in sequence and they are independent. In all of these respects they are analagous to gene mutations such as have been found in higher organisms. However, the frequency is high and the regularity with which each type gives characteristic proportions of variants is striking. Superficially, the situation here, as with the Salmonellas discussed by Dr. Dubos, seems quite different from the sort of mutation that has been studied by the Stanford group (10). The differences may be merely quantitative, but at this stage of our knowledge this is by no means certain. It is not impossible that there are nongenic factors

—*e.g.*, cytoplasmic factors—which control this sort of variation. Perhaps we are dealing with a situation more closely akin to cell differentiation than to genetic mutation. Although none of the facts known about color variation is irreconcilable with a theory of gene mutation, is it not possible that uncritical use of this concept at this time may tend to blind our sight to the operation of other discoverable mechanisms that may produce heritable bacterial variants or at least influence the production of such variants?

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## DISCUSSION

ALTENBURG: Could not your results be adequately explained on the basis of two facts which your experiments demonstrate: (1) that mutation from dark-red to bright-pink and reverse mutation take place at the rates you have found, and (2) that there is an occasional mutation to the white type in addition?

BUNTING: Not quite. These two mutations would not account for the pale-pinks of the first type, which upon analysis gave no white daughter colonies but only pale-pinks, bright-pinks, and dark-reds. I think at least three mutations would be required to account for the findings.

DAVIS: Dr. Bunting mentioned that certain types of bacterial variation may be "more akin to cell differentiation than to what is ordinarily meant by mutation." While lack of laboratory experience in either genetics or embryology makes me hesitate to take responsibility for expanding this point, it may be worth calling to the attention of bacteriologists and geneticists a statement by Joseph Needham in *Biochemistry and Morphogenesis* (p. 418). Here he points out that the main difference between genetics and embryology is that genetics studies inductors which remain within cell membranes, while embryology studies only more diffusible inductors. The application of this point of view to bacteriology suggests that bacteriologists may furnish a lamp to embryology rather than borrow a cane from genetics. Since study of the chemistry of inductors has been frustrated by the complexity of the embryologist's multicellular material, the stimuli to bacterial adaptation may be a type of inductor better suited for experimental analysis of the mechanism of cell differentiation. The pneumococcus-transforming substance appears to be a link between the

two fields. Indeed, bacterial variation may even provide a means of experimentally testing that hypothesis which would truly integrate these branches of cell physiology: namely, that cell differentiation itself involves alteration of genes.

This concept, while applicable to bacterial adaptations, seems less pertinent to apparently spontaneous variations. Dr. Dubos has noted that the regularity and high frequency of appearance of some of the latter type of variants suggests the classical genetic process of fusion with segregation of characteristics; in fact, evidence of bacterial fusion is presented by Dr. Dienes. But in the absence of any indication that fusion is a general property of bacteria, may one not consider the possibility of segregation without fusion? Such a mechanism could be provided by binary fission with asymmetrical apportionment of the genetic material. An alternative mechanism could be formulated on the assumption that the effect of a bacterial "gene" depends not only on its structure, but on its position in relation to the other genes. Internal rearrangements analogous to "crossing over," which would really not involve either fusion or segregation, could then account for the frequent, reversible, discontinuous modifications that have been observed. This armchair analysis is presented not with the conviction that either of these mechanisms is necessarily correct, but rather as an argument against the notion that bacterial inheritance must involve the patterns of classical genetics.

BUNTING: I am entirely in agreement with the point of view discussed by Dr. Davis, and I believe that the color variations which I have described lend themselves well to further analysis of mechanisms of bacterial inheritance.

# INDUCED MUTATIONS IN BACTERIAL VIRUSES[1]

## M. DELBRÜCK AND W. T. BAILEY, JR.

In another paper of this Symposium, to which ours is closely related and to which we refer the reader for a description of material and for terminology, Dr. Hershey (4) has described a variety of spontaneously occurring mutations of bacterial viruses. One class of mutations affects the type of plaque. These mutations occur in only one group of serologically related viruses, the group to which belong T2, T4, and T6. The most conspicuous of these mutations is the *r* mutation. Our observations are concerned exclusively with this *r* mutation. We have seen also some of the other mutations which affect the type of plaque and which Hershey has described, but we have not made systematic experiments concerning them.

We have infected bacteria simultaneously with mixtures of wild type and *r* mutant of the viruses T2, T4, and T6, and have investigated the yields of virus from such mixedly infected bacteria. These experiments are a sequel to previous studies of mixed infections with pairs of different viruses. The chief result of the studies to be reported here is the fact that the yield of virus from mixedly infected bacteria may contain a high proportion of one or more new types of virus—i.e., of a type that was not used for the infection. In all cases the new types exhibit combinations of the genetic markers of the infecting types.

## MUTUAL EXCLUSION

We will begin with a recapitulation of earlier work on mixed infections (3, 6, 2). The chief finding of these earlier studies was the mutual-exclusion effect. It was found that any mixedly infected bacterium yields upon lysis only one of the infecting types of virus. The other virus does not multiply; even the adsorbed particles of the excluded type are not recovered upon lysis. Which one of the two types of virus used for infection is excluded and which one multiplies depends on the pair used and on the conditions of the experiment, such as timing and multiplicity of infection. For the pair T1, T2 the virus T1 is always excluded except when it is given a head start of at least four minutes (3). If T1 is added more than four minutes earlier than T2, then T2 will be excluded in an appreciable proportion of the infected bacteria. Lysis of any one bacterium always occurs after a time interval corresponding to the latent period of intracellular virus multiplication of the virus type which does multiply in that particular bacterium.

A similar situation was encountered (2) in the study of the pair T1, T7. Here, too, the mutual-exclusion mechanism operates perfectly; in practically every bacterium either one or the other of the two viruses is excluded from multiplication. The exclusion powers of these two viruses are nearly balanced. In a bacterial culture simultaneously infected with T1 and T7 there is a clean split into T1 yielders and T7 yielders, the two types occurring with comparable frequency.

During a closer study of this pair it became evident that the excluded virus is not without effect on the course of events. The excluded virus may reduce the number of virus particles liberated upon lysis of the bacterium. This has been called the depressor effect (2).

A cursory survey of other pairs of virus particles seemed only to confirm these findings and, in particular, seemed to point to the mutual-exclusion effect as a very general phenomenon.

In these earlier investigations an attempt was made also to test whether mutual exclusion occurs when a bacterium is infected with two particles of the *same* kind. Such an assumption ("self-interference") seemed to suggest itself from the observation that bacteria infected with several particles of one kind are lysed after exactly the same latent period as are bacteria infected with only one particle. The test of mutual exclusion requires that one find out whether the yield of virus from any one bacterium is the offspring of only one or of several of the infecting particles. To make such a test one must be able to differentiate the offspring of the various infecting particles; in other words, one has to mark the infecting particles with hereditary markers. One is thus naturally led to the study of mutual exclusion between a virus and one of its mutants.

The first attempt in this direction was made by Luria (5), who studied interference between T2 and T2*h*. The difficulty with this pair lies in the fact that no indicator strain resistant to T2*h* and sensitive to T2 is available. Luria succeeded nevertheless in showing that a large proportion of the bacteria infected with T2 *and* T2*h* did not liberate any T2*h*. At least a partial functioning of the mutual-exclusion mechanism seemed to be indicated by these results.

## THE BREAKDOWN OF MUTUAL EXCLUSION FOR THE PAIRS (T2r+, T2r) AND (T4r+, T4r)

The first definite indication of a new phenomenon came in March, 1945, when Hershey tried mixed infections with a wild-type strain (T2) and its *r*

mutant. Hershey observed that the mixedly infected bacteria give rise to mottled plaques, and he verified that the mottled plaques contain a mixture of the two types used for infection. Dr. Hershey communicated his discovery to us and we have since been following this promising lead.

### Mixed Infections with T2r⁺, T2r

Hershey's finding that the majority of the mixedly infected bacteria give mixed yields was confirmed by two methods; viz., (1) by plating mixedly infected bacteria before lysis, (2) by plating single bursts after lysis (1).

In these experiments the infecting doses of wild type were slightly higher than those of the r mutant. Each kind of virus was in at least threefold excess over the bacteria. In the case of simultaneous infection, about one-third of the bacteria gave pure wild-type bursts. Most of the remaining bursts were mixed. These mixed bursts contained wild type and mutant in all proportions. On the average, however, wild type was predominant. The predominance may be due to an inherent advantage of wild type, or it may be due to the fact that in these experiments the infecting doses of wild type were slightly greater than those of the mutant. We have found that the wild type of this strain of virus is somewhat more rapidly adsorbed than its r mutant, and the predominance of wild type in the bursts may be due in part to the more rapid adsorption of the wild type.

If the two viruses are not given simultaneously, the ratios are shifted in favor of the virus which precedes. Thus, if wild type precedes by six minutes, almost all bursts are pure wild type. If the r mutant precedes by six minutes, there is a majority of pure r bursts and mixed bursts, but there is still a fair proportion of pure wild type.

### Mixed Infections with T4r⁺, T4r

The results for this pair were similar to those for mixed infections with wild type and r mutant of T2, with the following minor differences:

(1) A greater proportion of the single bursts showed mixed yields (23 out of 25).

(2) The r mutants predominated in the mixed bursts, although all proportions were encountered. Fig. 1 shows the correlations between wild type and r mutant in the individual bursts of one large experiment, in which sixty samples were plated for bursts.

For this pair, too, it was found that wild type is adsorbed slightly more rapidly than is its r mutant.

These experiments substantiated Hershey's findings. They showed, moreover, that in the bacteria giving mixed yields all proportions of wild type to r mutant could be found. The results seemed to prove an almost complete breakdown of the mutual-exclusion mechanism, nearly every bacterium yielding virus particles of both the infecting types. However, modifications of the experimental set-up to be reported presently revealed unexpected new fea-

tures, which throw the interpretation of Hershey's experiment into doubt.

It may be recalled that the first experiments on mixed infection had been undertaken in the hope of obtaining lysis of bacteria at an intermediate stage of intracellular virus multiplication. Our expectation had been that in mixed infection with (T1, T2) the bacteria would be lysed after 13
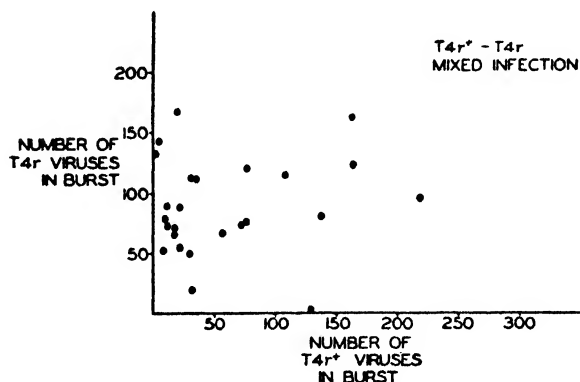


FIG. 1. T4r⁺ and T4r content of individual bursts of bacteria infected with these viruses. Each dot represents one burst. The abscissa is the T4r⁺ content, the ordinate the T4r content.

minutes, the latent period of T1, and that thus an intermediate stage in the multiplication of T2 would be revealed. T2 by itself does not lyse the bacteria until 21 minutes after infection. This hope had been frustrated when mutual exclusion was discovered. Hershey's discovery of an apparently complete breakdown of the mutual-exclusion mechanism for infections with the pair (T2r⁺, T2r) revived the hope of studying intermediate stages of intracellular multiplication of viruses. However, the study of pairs like (T2r⁺, T2r) suffers from the handicap that indicator strains are not available for obtaining separate counts of the two types. Instead, one has to rely on plaque appearance. The majority of the plaques are clearly differentiated, but there are always a few plaques whose classification is a little uncertain. These would require subculture for verification, a very laborious procedure when large numbers are involved. For this reason we eventually (October, 1945) decided to try the pairs (T2, T4), (T6, T4), and (T2, T6). These viruses are closely related to each other serologically and morphologically, but they are otherwise independent wild types, and indicator strains which sharply distinguish between them are available. The r mutation was used as an additional genetic marker.

### THE BREAKDOWN OF MUTUAL EXCLUSION FOR THE PAIRS (T4, T2), (T4, T6), AND (T2, T6)

In Hershey's case of mixed infection with wild type and r mutant, a breakdown of mutual exclusion is indicated by the appearance of mottled plaques

when mixedly infected bacteria are plated. For pairs with host-range differences another criterion can be employed, the appearance of clear plaques in platings on mixed indicator strains (2). Any bacterium that liberates at least one particle of each of the infecting types can lyse both indicator strains, while any bacterium that liberates only particles of one of the infecting types will form a plaque which is overgrown by the indicator strain for the other type and which will therefore be turbid. A comparison of plaque counts on mixed indicators and on strain B gives the fraction of the bacteria with mixed yields—i.e., the fraction of the bacteria in which the mutual-exclusion mechanism failed to operate.

Table 1 lists the fraction of mixed yielders for a variety of combinations between the wild types and

TABLE 1. THE BREAKDOWN OF THE MUTUAL-EXCLUSION PRINCIPLE

| Infecting Pair | | % Mixed Yielders |
|---|---|---|
| T6 | T4 | |
| $r^+$ | $r^+$ | Few |
| $r$ | $r$ | 10 |
| $r$ | $r^+$ | 20 |
| $r^+$ | $r$ | 12 |
| T2 | T4 | |
| $r^+$ | $r^+$ | 60–90 |
| $r^+$ | $r$ | 60–90 |
| $r$ | $r^+$ | 80 |
| T2 | T6 | |
| $r^+$ | $r$ | 80 |

$r$ mutants of T2, T4, and T6. The breakdown of the mutual-exclusion mechanism is particularly marked for the combinations involving T2. These findings would seem to fit well with Hershey's findings, and to suggest the generalization that mutual exclusion operates the more perfectly the more dissimilar the two infecting viruses. Wild type and $r$ mutant give no mutual exclusion, serologically closely related viruses give partial mutual exclusion, and unrelated viruses give complete mutual exclusion.

### The Nature of the T2 Particles Liberated by Mixedly Infected Bacteria Involving T2

A paradoxical feature occurred in all the combinations involving T2. When mixedly infected bacteria were plated, the number of *clear* plaques on mixed indicators was in all cases much greater than the number of plaques on the indicator for T2. The number of clear plaques *on mixed indicators* gives the number of bacteria which liberate a mixture of the infecting types. The number of plaques *on the T2 indicator* gives the number of bacteria which liberate T2, irrespective of whether or not such a bacterium also liberates the other type. The latter

plaque count comprises two classes, the mixed yielders plus the pure T2 yielders. The mixed indicators show only one of these classes, the mixed yielders. It follows that the count on mixed indicators should always be smaller than, or at most equal to, the count on the T2 indicator, contrary to the actual finding.

The possibility was considered that the clear plaques found on mixed indicators might be due, not to a mixed yield of T2 and T4 particles, but to host-range mutants. Conceivably, host-range mutants might arise during the growth of the plaques and these mutants might be responsible for the lysis of both indicator strains. It must be realized that mixed indicators constitute an ideal enrichment medium for host-range mutants.

This possibility was ruled out by two tests. First, the viruses in question were plated separately on the same pair of mixed indicators. No clear plaques were found. This shows that host-range mutants in sufficient number to cause clear plaques on mixed indicators do not occur when the viruses are plated separately. Second, the contents of ten of the clear plaques from platings of mixedly infected bacteria on mixed indicators were analyzed for their virus content. Each of these plaques contained a mixture of particles with host ranges characteristic of the infecting types, and no particles with extended host range.

It follows that the clear plaques on mixed indicators are due to genuine mixed yields of T2 and T4 particles. The low count of T2 yielders on the indicator for T2 must mean that a considerable proportion of the putative T2 particles liberated from mixedly infected bacteria fail to form plaques on the T2 indicator. The T2 particles used for infection in these experiments do not have this property; they register with the same efficiency on the T2 indicator as on mixed indicators. The low efficiency of plating of the T2 particles in the yields from mixedly infected bacteria is not a hereditary property. Their progeny registers with the same efficiency on the T2 indicator as on mixed indicators.

It might be mentioned in passing that there are many T2 indicator strains of the type B/4 on which the normal T2 exhibits a low efficiency of plating. In fact the strain of B/4 employed in these experiments is unique in registering T2 with full efficiency of plating. This strain was isolated by Dr. Hershey. The question arose whether the low efficiency of plating of the normal T2 on the other strains of B/4 is also increased by the addition of B/2. Experiments showed that this is not the case. Therefore, low efficiency of plating of the T2 particles liberated from mixedly infected bacteria must be caused by a specific property of these particles.

The findings may be summarized as follows. A large proportion of bacteria mixedly infected with T4 and T2, or T6 and T2, give mixed yields. The liberated T2 particles have a low efficiency of plating on the T2 indicator but not on mixed indicators.

The low efficiency of plating of these particles on the T2 indicator is not a hereditary property.

More experiments will be needed to clarify this situation. Specifically, the role of the second indicator strain in raising the efficiency of plating of the liberated T2 particles has to be clarified. This indicator strain by itself is totally resistant to T2. Preliminary experiments have not given any clue to the factor contributed by the second indicator strain.

## INDUCED MUTATIONS

We now turn to the principal point of this paper, the occurrence in mixed infections of induced mutations at the *r* locus. In the experiments described in the two preceding sections, the mixedly infected bacteria were regularly plated on strain B, on each of the indicator strains separately, and on mixed indicators. In some of these experiments one of the infecting viruses was genetically marked by using the *r* mutant instead of the wild type. It was noted that the platings on one or the other of the indicator strains, or on both, gave a high proportion of mottled plaques. For instance, in a mixed infection with the viruses (T2$r^+$, T4$r$) the plating on B/2 gave plaques the majority of which had mottled halos, indicating that the majority of the bacteria had liberated a mixture of wild-type and *r*-type particles which could attack B/2. Wild-type particles able to attack B/2 had not been used in this experiment. The wild-type particles, therefore, represented a new type, created during the mixed infection.

The creation of wild-type particles with the host range characteristic of T4 was verified in three ways. First, by analyzing the contents of the mottled plaques. These analyses confirmed the assumption of the presence of a mixture of T4$r^+$ and T4$r$ particles. Second, by plating on B/2 after lysis. These plates, too, showed the presence of T4$r^+$, though in smaller proportion, indicating that induced mutants occur in small numbers, though they occur in the yields of the majority of the mixedly infected bacteria. Third, by plating single bursts on B/2. Small numbers of induced mutants were found in the majority of the bursts, confirming the inference of the previous test.

*A priori,* this new type could have arisen as a modification of either one of the infecting types: either as a modification of T4$r$ by a mutation

    T4$r$ $\longrightarrow$ T4$r^+$ (under the influence of T2$r^+$),

or as a modification of T2$r^+$ by a mutation

    T2$r^+$ $\longrightarrow$ T4$r^+$ (under the influence of T4$r$).

The first assumption implies a mutation at the *r* locus, the second assumption a mutation at the genetic site (or sites) determining host range. We prefer the first hypothesis for three reasons. First, the mutation at the *r* locus is known to occur spontaneously and is known to require only one step. Second, a change from the host range characteristic

for T2 to that characteristic for T4, as implied in the second assumption, may be expected to require several mutational steps, since T2 and T4 are independent wild types, and since at least two phenotypic changes are involved—namely, loss of activity on B/4 and gain of activity on B/2. Third, when the new type was tested serologically, it was found to be indistinguishable from the infecting type, with the same host range. We therefore believe that the new types arise by a mutation at the *r* locus, without change of host range.

Table 2 is a summary of the combinations of in-

TABLE 2. MIXED INFECTIONS YIELDING INDUCED MUTATIONS

| Infecting Pair | | Induced Types |
|---|---|---|
| T6 | T4 | |
| $r^+$ | $r^+$ | none |
| $r$ | $r$ | none |
| $r$ | $r^+$ | T6$r^+$, T4$r$ |
| $r^+$ | $r$ | T4$r^+$ |
| T2 | T4 | |
| $r^+$ | $r^+$ | none |
| $r^+$ | $r$ | T2$r$, T4$r^+$ |
| T2 | T6 | |
| $r^+$ | $r$ | T2$r$ |

fecting types which have been tested for the occurrence of induced mutations at the *r* locus. The data point to three generalizations:

(1) Mutations occur from wild type to *r* type or from *r* type to wild type.

(2) Mutations occur only if one of the infecting types is wild type, the other *r* type. When both the infecting types are wild type, or both *r* type, no mutations are found.

(3) In the same mixed infection both infecting types may be changed, wild type to *r* type, and *r* type to wild type.

A discussion of possible theoretical interpretations of these findings does not seem warranted at this point, since our studies are far from complete. Perhaps one might dispute the propriety of calling the observed changes "induced mutations." In some respects they look more like transfers, or even exchanges, of genetic materials. We do not pretend to be able to put forward convincing arguments for either point of view.

A comment might be added with respect to Hershey's original discovery of mixed yields of wild type and *r* type. We now know that mutual exclusion may break down in infections with closely related viruses. Hershey's finding may therefore be interpreted as a lack of mutual exclusion. On the other hand, we know that in mixed infections with wild type and *r* type induced mutations do occur. To explain Hershey's findings one might assume, therefore, that only one of the infecting types multiplies while the other type induces mutations in it. A much closer study of the interrelations between

the breakdown of mutual exclusion and the occurrence of induced mutations will be necessary to settle this ambiguity. For this purpose detailed studies of the contents of single bursts, and of the numerical relations between the different types of viruses in such bursts, should prove of great value.

## SUMMARY

We will briefly retrace in historical order the steps that have led to our present state of knowledge regarding mixed infections of bacteria with bacterial viruses.

Mixed infections with pairs of *unrelated* viruses, like (T1, T2) or (T1, T7), result in mutual exclusion. Only one of the infecting types multiplies, the other is lost (*3, 6, 2*).

The excluded virus may greatly reduce the yield of successful virus (depressor effect) (*2*).

Mixed infections with wild-type and *r*-type particles of the *same* strain do not, apparently, give rise to mutual exclusion (Hershey, *4*).

Mixed infections with pairs of *related* viruses of the group T2, T4, T6 give partial mutual exclusion. The T2 particles liberated in mixed infections of this type exhibit certain nonheritable peculiarities, the nature of which has not yet been ascertained.

Mixed infections with pairs of the group T2, T4, T6, in which one of the pair is used in the wild-type form, the other in the *r*-type form, give rise to the liberation of new types, which can be characterized as mutants of the infecting types. The mutations occur at the *r* locus.

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# MUTATIONS IN BACTERIA INDUCED BY RADIATIONS

## M. DEMEREC AND R. LATARJET

Although direct genetic tests that might prove the occurrence of mutations in bacteria cannot be made, it seems reasonable to assume from the accumulated indirect evidence that in this group of microorganisms changes that are comparable to mutations in higher organisms do occur. As might be expected, such changes affect a great variety of characteristics; in fact, mutants have been found representing all types of character for which a search has been made. For illustrations of a wide variety of bacterial mutants we do not have to go beyond the papers presented at this Symposium, where we have heard discussions of mutations affecting color (Bunting), enzymatic systems (Lwoff), biochemical characteristics (Tatum), and resistance to phages, drugs, and antibiotics (Luria).

In this paper we will consider only the specific mutations in *Escherichia coli* that are responsible for resistance to a certain bacteriophage. It has been shown earlier that such mutations can readily be induced by both the ultraviolet rays and X-rays (*4*). Our experiments have revealed that mutations in *E. coli* resulting in resistance to bacteriophages provide an exceptionally favorable opportunity for quantitative studies of the genetic effects of ultraviolet radiation and for comparative studies of the genetic effects produced by ultraviolet rays and X-rays. In fact, this material appears so far to be unique for the study of the relation between ultraviolet dosage and induced mutation rate. Such a study could not be carried out on Drosophila, because of excessive absorption of the rays by the tissues and by the extranuclear constituents of the cells. Even in the case of maize (*12*), the absorption of ultraviolet radiation by the pollen wall and contents was found to be great enough to cause large differences in the intensity of the radiation penetrating to the site of the nucleus in different pollen grains. Studies made on fungi (*7*) revealed a complex situation, for which an entirely satisfactory explanation has not yet been found. Even the use of the small microconidia of Neurospora in irradiation experiments has not made the picture any clearer (*8*). In all experiments with fungi where quantitative data for mutation rate in relation to dosage are available, they show two features—namely, considerable variability in repeated experiments, and an increase in the mutation rate with increased dosage followed by a sharp drop after a certain level has been reached.

## MATERIAL

Two lines of the B strain of *E. coli* were used in our experiments. One of them is the regular B strain, and the other the B/r line derived from B by Witkin (*13*). B/r is characterized by resistance to ultraviolet and X-rays, and because of this feature it was selected for our experiments. Since it can stand treatment with higher doses of both radiations than the B strain, it was anticipated that irradiation-induced increases in mutation rate would be more easily detected in this line. B/r was used in most of our experiments.

Bacteriophage T1, known also as "alpha" and "P28," was used to isolate mutants resistant to this phage.

## NOMENCLATURE

The system of nomenclature developed recently for describing genetic studies of bacterial resistance will be used in this paper. According to this system, resistant mutants are designated by the symbol of the sensitive strain (B in the case of our experiments) followed by a crossbar and the symbol describing the mutant. For example, B/1 is the symbol for a mutant derived from B that is resistant to the phage known as T1; B/r symbolizes a mutant of B that is resistant to radiations; and B/r/1 symbolizes a mutant derived from B/r that is resistant to phage T1—or, in other words, a two-step mutant of B resistant both to radiation and to phage T1.

In this paper we will use the symbols B, B/r, B/1, and B/r/1.

## METHODS

In all experiments reported here, bacteria were cultured in a synthetic medium (known as M-9) because this medium is almost transparent to ultraviolet radiation. M-9 is the laboratory name for an ammonium chloride-glucose-phosphate buffer medium developed by E. H. Anderson (*1*).

Each day a new culture was started, using as an inoculum a small number of bacteria taken from a stock culture grown on a broth-agar slant. In order to reduce the chances of genetic variability in the stock culture, the same slant was used as the source of inoculum for four to six weeks and was kept meanwhile in a refrigerator.

All assays for determination of the concentration of living bacteria in cultures were made by plating a sample of suitably diluted culture onto broth-agar plates, and counting the number of colonies after 24 hours of incubation at 37° C.

For determining the number of B/1 or B/r/1 mutants, two methods were used. When a determination was to be made of mutants already present

in the culture or showing up immediately after irradiation (zero-point mutants), a sample of culture containing a known number of bacteria was plated onto a Petri dish previously coated with about $10^8$ particles of phage. During 48 hours of incubation at 37° C. all sensitive bacteria were eliminated by the phage, while the resistant bacteria survived and formed colonies, which were then counted.

Another method was used for determining the number of delayed mutants—those that show up after the bacteria have passed through a number of divisions following irradiation. In such cases, a known number of bacteria was plated onto Petri dishes; these were incubated for the period of time necessary for the bacteria to pass through the desired number of divisions, and then the phage was sprayed over the surface of the plates as a fine mist (4). The main advantage of this method is that the positions of the bacteria in a growing colony are not disturbed by application of the phage, and that therefore all mutations occurring within a certain period are represented by single colonies, no matter how many mutant bacteria have been formed by division of the original mutant individual.

In critical experiments, particularly when the number of mutants was small, sample tests were made to establish whether or not bacteria of surviving colonies were mutants. These bacteria were tested with phages T1 and T2. If they were sensitive to T2, this made it probable that they belonged to strain B and were not contaminants, while resistance to T1 indicated that they were mutants. Colonies of the B strain have characteristic semirough markings, by which it is possible to distinguish them from contaminants. To minimize air contamination during manipulation of plates, 0.1 ml. of an 0.4% aqueous solution of gentian violet was added to each liter of the agar medium before the plates were poured.

### TECHNIQUE OF IRRADIATION
#### Ultraviolet Rays

The source was a General Electric germicidal lamp, about 80% of whose ultraviolet output consists of the resonance radiation of mercury, wave length 2537 A. As this wave length is especially efficient both in bacterial killing (5) and in the production of mutations (7), it is considered that more than 90% of the effects we were dealing with were due to this wave length. It can be assumed, therefore, that in our experiments we were using an almost monochromatic ultraviolet beam.

It was very important to have a reliable measurement of the dosage; and for this reason we made a careful study of the output of our lamp, the voltage being kept constant at 110 V.

The absolute value of the ultraviolet flux at one meter from the center of the bulb was first obtained by comparison with a lamp of the same type previously calibrated in absolute units by Dr. A. Hollaender. The value thus obtained was checked by means of a sensitive biological test; namely, the inactivation of phage T2. The relation between this inactivation and dosage had previously been determined in absolute units with an ultraviolet standard (10). The two values agreed with a precision of 2%, giving an intensity of 5.6 ergs $\times$ mm.$^{-2}$ $\times$ sec.$^{-1}$ for 2537 A. This value was checked several times during the course of the experiments.

The distribution of the intensity according to distance from the bulb and distance from the normal axis was measured by means of a small-surface photocathode (1 mm.$^2$) operating under such conditions that the photoelectric current was proportional to the radiant flux received. In most of our experiments we irradiated at a distance of 56 cm. from the source, where the intensity was equal to 1000 ergs $\times$ mm.$^{-2}$ $\times$ min.$^{-1}$. At this distance the intensity was uniform on the whole irradiated sample; that is, on a Petri dish 9 cm. in diameter.

The bacteria, suspended in a transparent medium, either saline or synthetic M-9, were irradiated in open Petri dishes. The depth of the suspension was about 1 mm. Its absorption was negligible, as long as the concentration of bacteria did not exceed $10^8$ per ml. The dosage was therefore directly determined by the exposure time. As the full-grown 48-hour culture in aerated M-9 reached a concentration of about $5 \times 10^9$ bacteria per ml., diluted suspensions were obtained by the addition of saline, rather than M-9, in order to avoid any futher growth during irradiation and assays. It had previously been determined that irradiation of M-9 and of saline suspensions gives identical results.

When very high dosage was required, producing a high rate of killing, we were obliged to irradiate the full-grown culture directly, in order to obtain enough survivors for the detection of mutants. Such suspensions, even in thin layers, absorb a large amount of the incident ultraviolet beam, by true absorption and by diffusion. Continuous shaking of the Petri dish helped to make the dosage uniform for the whole sample; but then the dosage could not be determined directly, because the diffusion prevented photometric measurement. In order to obtain this value, then, we determined in every experiment the rate of survivors, and the dosage was read from the survival curve previously prepared from experiments in which transparent suspensions had been used.

#### X-rays

We used the X-ray machine of the Memorial Hospital in New York City, which was kindly placed at our disposal by Mr. L. D. Marinelli and Miss E. Focht. The source consisted of 2 tubes with tungsten anticathodes, placed face to face 30 cm. apart and operating under 180 kV and 25 mA. The total radiation was filtered only by the glass of the X-ray tubes. It gave, when absorbed in copper,

an H.V.L. of 0.19 mm., which corresponds to an average wave length of 0.35 A. The intensity of the radiation, measured between the two anticathodes at a point equidistant from each, was 2050 roentgens per minute. The bacterial suspension, 2 cm. in depth, was placed at this point, in a thin-walled pyrex tube 1 cm. in diameter.

Under such conditions, the synthetic medium M-9 containing the bacteria behaves approximately as a water layer 1 cm. in depth exposed to a single beam of 2050 r per minute. In spite of the fact that the unfiltered beam is highly heterogeneous, one can obtain an approximate idea of the absorption by considering the behavior of the radiation of average wave length (0.35 A). Such a radiation, corresponding to a voltage of 35 kV, has an absorption coefficient in water of $\mu = 0.35$ cm.$^{-1}$. Assuming that the penetration of the rays into the suspension follows an exponential law, the average dose, Dave, produced by the surface dose, D, is obtained from the formula:

$$\text{Dave} = \frac{D\ (1 - e^{-\mu x})}{\mu x} = 0.84\ D,$$

where $\mu = 0.35$ and $x = 1$. The secondary electron in water has an average path of 25 microns, producing about 1000 ion pairs. The average number of ionizations, N, produced in one cubic micron of the suspension (taken as equivalent to air of unit density) by a given dose, D roentgens, of that radiation is obtained from the formula:

$$N = \frac{10^{-12}}{0.001293 \times 4.7 \times 10^{-10}} \times \frac{D(1 - e^{-\mu x})}{\mu x} = \frac{1.6\ D(1 - e^{-\mu x})}{\mu x} = 1.38\ D.$$

For instance, a dose of 10,000 r produces an average of 13,800 ionizations per cubic micron.

## DESCRIPTION OF EXPERIMENTS

A small fraction of the B/1 and B/r/1 mutations induced by X-ray and ultraviolet irradiation shows up before the bacteria have had a chance to divide (4). These will be called "zero-point mutations," and can be detected by applying phage to the bacteria immediately after irradiation—that is, before any bacterial divisions have occurred. Another and much larger fraction of the mutations shows up as the bacteria pass through several divisions. The mutation rate per bacterial generation is highest during the period when the bacteria are passing through the first few divisions after treatment. The rate then begins to decrease, and reaches a normal level (level accounted for by spontaneous mutations) when the bacteria have passed through approximately 13 divisions subsequent to treatment (4). The total number of mutations resulting from the irradiation can be determined, therefore, by making observations at the point when the muta-

tion rate reaches a normal level; and the term "end-point mutations" will be applied to this total number. "Zero-point" and "end-point" are arbitrary terms derived from laboratory usage. To obtain the zero-point value, phage was applied at the beginning of an experiment (0 hours), and to obtain the end-point value phage was applied at the end of an experiment.

The experiments performed during this study were too numerous to be reported in detail. Results are shown graphically in Figs. 1 and 2. In order to illustrate experimental procedure, we shall describe here two experiments: one to determine the "zero-point" (immediate) mutation rate, and the other the "end-point" (total) mutation rate. In both these experiments the bacteria were treated with ultraviolet radiation.

### Determination of the Zero-Point Mutation Rate

In each experiment of this series, tests with several doses were included, so that the results of any one experiment indicated a general trend of the relation between dosage and rate of induced mutations. The experiment described below has been chosen as an example to illustrate operations required when both low and high doses are used. Since very few bacteria survive treatment with a high dose, it becomes necessary after such treatment to concentrate the bacterial suspension by centrifugation so that a number of living bacteria adequate for the detection of mutants can be obtained in the test sample. The experiment is described here in detail.

*December 18.* About 1000 B/r bacteria, taken from a slant, were inoculated into 250 ml. of M-9 synthetic medium, then incubated at 37° C. with aeration.

*December 20.*
(A) Assay of the 48-hour full-grown culture:
   (1) To determine concentration of bacteria —by plating suitable dilutions and counting colonies. (Assay *1*)
   (2) To determine concentration of spontaneous B/r/1 mutants—by plating 0.1 ml. each on plates previously coated with about $10^8$ particles of T1 phage. (Assay *2*)
(B) Irradiation, assays of survivors, and centrifugations: 7 ml. of the B/r culture, containing about $4 \times 10^9$ bacteria per ml., were poured into each of 24 Petri dishes (9 cm. in diameter); then each dish was irradiated, with intermittent shaking. The intensity of irradiation was equal to 1000 ergs $\times$ mm.$^{-2}$ $\times$ min.$^{-1}$. After irradiation, all the samples that had received the same dosage were combined, for assay of survivors and for centrifugation. Four different doses were given:
   (1) *1 minute*—2 Petri dishes. Assay *3*.

(2) *2 minutes*—2 Petri dishes. Assay *4*. Centrifuged once, reducing 13 ml. to 1.3 ml. Assay (*5*) of the centrifuged suspension.

(3) *3 minutes*—10 Petri dishes. Assay *6*. Centrifuged twice, reducing 70 ml. to 7 ml., and again 7 ml. to 1 ml. Assay (*7*) of centrifuged suspension.

(B) *Mutants (2)* 15 B/r/1 mutants per $10^8$ bacteria.

(C) *First exposure (3)* $1.06 \times 10^9$ B/r per ml. Hence the rate of survival was 25%. From the survival curve the dose was read as 800 ergs $\times$ mm.$^{-2}$. The number of bacteria plated on phaged plates in tests for mutants was $1 \times 10^8$ per plate.
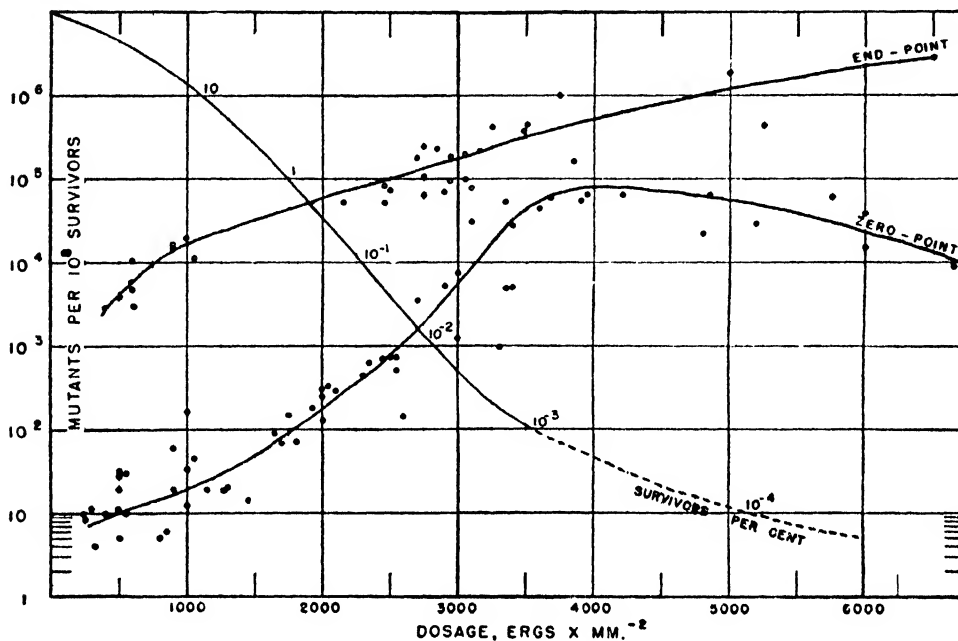


Fig. 1. Zero-point and end-point mutants induced by ultraviolet 2537 A in B/r, and ultraviolet survival curve of B/r (semilogarithmic plot). The dots refer to experimental 0-point results, and the circles to experimental end-point results. The curves have been drawn to fit these points.

The survival curve is of a multiple-hit type. The zero-point mutation rate increases very rapidly with increased dosage, up to a maximum corresponding to a dose of 4000 ergs x mm.$^{-2}$, then seems to drop slowly. The end-point mutation rate increases slowly with increased dosage, but does not show any drop.

(4) *4 minutes*—10 Petri dishes. Assay *8*. Centrifuged twice, reducing 70 ml. to 7 ml., and again 7 ml. to 1 ml. Assay (*9*) of the centrifuged suspension.

(C) Plating for assays of mutants: The numbers of B/r/1 mutants were determined in the irradiated (1) and centrifuged (2, 3, and 4) suspensions, by plating for each set 0.1 ml. on each of 8 agar plates previously coated with about $10^8$ T1 phage particles. The plates were incubated for 48 hours at 37° C., and the numbers of B/r/1 colonies were counted.

*December 21.* Scoring of the assays: After 24 hours of incubation at 37° C., the assays were scored. They gave the following results:

(A) *Initial culture (1)* $4.26 \times 10^9$ B/r per ml.

(D) *Second exposure (4)* $6.8 \times 10^7$ B/r per ml. Rate of survival, 1.6%. Dose, 1650 ergs $\times$ mm.$^{-2}$. Centrifuged suspension (*5*), $4.3 \times 10^8$ per ml. Number of bacteria plated on phaged plates in tests for mutants, $4.3 \times 10^7$ per plate.

(E) *Third exposure (6)* $2.17 \times 10^6$ B/r per ml. Rate of survival, $5 \times 10^{-2}$ %. Dose, 2550 ergs $\times$ mm.$^{-2}$. Centrifuged suspension (*7*), $1.65 \times 10^8$ per ml. Number of bacteria plated on phaged plates in tests for mutants, $1.65 \times 10^7$ per plate.

(F) *Fourth exposure (8)* $3 \times 10^5$ B/r per ml. Rate of survival, $7 \times 10^{-3}$ %. Dose, 3000 ergs $\times$ mm.$^{-2}$ Centrifuged suspension (*9*), $2.2 \times 10^7$ B/r per ml. Number of bacteria plated on phaged plates in tests for mutants, $2.2 \times 10^6$ per plate.

*December 22.* Scoring of mutants:

| Plate No. | No. of mutants at various doses (ergs $\times$ mm.$^{-2}$) | | | |
|---|---|---|---|---|
| | a 800 | b 1650 | c 2550 | d 3000 |
| 1 | 13 | 43 | 118 | 276 |
| 2 | 17 | 58 | 106 | 236 |
| 3 | 10 | 46 | 99 | 270 |
| 4 | 19 | 50 | 120 | 80 |
| 5 | 15 | 43 | 138 | 137 |
| 6 | 12 | 45 | 95 | 135 |
| 7 | 20 | 30 | 119 | 111 |
| 8 | 14 | 43 | 141 | 123 |
| 9 | | | 162 | 161 |
| Average number of mutants per plate | 15 | 44.7 | 122 | 170 |
| Number of bacteria plated per plate | $7.6 \times 10^7$ | $4.3 \times 10^7$ | $1.65 \times 10^7$ | $2.2 \times 10^6$ |
| Number of mutants per $10^8$ bacteria | 20 | 104 | 740 | 7735 |
| Same after deducting spontaneous mutants | 5 | 91 | 725 | 7720 |

The numbers listed in the bottom line are zero-point mutants induced by the doses indicated at the head of the table.

### Determination of the End-Point Mutation Rate

The fundamental assumption for these experiments is that, following irradiation, the mutation rate per bacterial generation decreases after the first few bacterial divisions and reaches the normal level of spontaneous mutation when the bacteria have passed through approximately 13 divisions. The end-point mutation rate, therefore, may be determined by ascertaining when the bacteria on the plates have passed through 13 divisions, and then spraying the phage over the plates to isolate mutants. The end-point mutation rate may also be determined by applying phage at intervals during the period when it is expected that the bacteria may be at the desired stage of division, and then deciding from the number of mutant colonies counted when the end point has been reached. During the time when delayed mutations are still appearing, the increase in the total number of mutants is due to both delayed and spontaneously occurring mutations; but after delayed mutations have stopped appearing, the increase in the total number of mutants is due to spontaneous mutation alone. Since the spontaneous mutation rate is low (about one-half to one per $10^8$), it is possible to determine approximately when delayed mutations stop, if data on the total number of mutants are available for the critical period. At the point when delayed mutations cease to appear, the increment in the total number of mutants drops and reaches the level determined by the rate of spontaneous mutation.

It is known that radiations delay the beginning of division in the treated bacteria, but that once irradiated bacteria start dividing they continue to
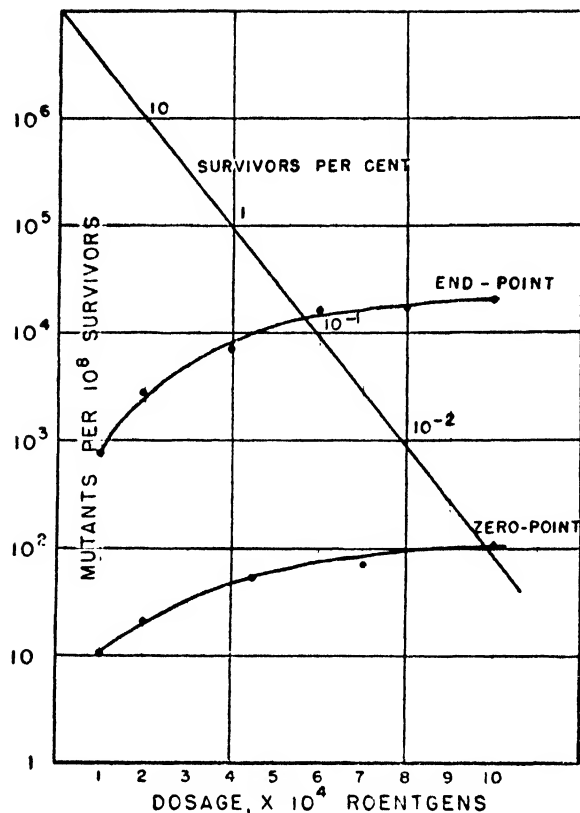


Fig. 2. Zero-point and end-point mutants induced by X-rays in B/r, and X-ray survival curve of B/r (same semi-logarithmic plot as Fig. 1). Dots and circles show experimental results.

The survival curve is of a one-hit type. The zero-point mutation rate is proportional to the dosage (see Fig. 3). The ratio between the end-point and zero-point mutation rates remains about the same, 100:200. The dosage given is that at the surface; to obtain the average dose in suspension the correction factor 0.84 should be used.

multiply regularly. After irradiation there is an extension of the lag period in resting bacteria as well as a delay of the start of division in growing bacteria. In both cases, the delay is greater after treatment with high doses. Therefore, in order to determine when the plated bacteria have passed through 13 divisions, it is necessary to determine when they begin to divide. After that point B and B/r pass through one divisional cycle, on the average, every 19-20 minutes, if grown either in broth or on a broth-agar medium. Previous observations (4) have indicated that irradiated B/r bacteria begin to divide at approximately the same time whether they are placed in broth or plated on broth-agar. Since it is simpler to determine when the bacteria begin to divide if they are grown in broth, all of our determinations of the extent of the lag period following irradiation were made on broth cultures, by the plating method.

To determine the end-point number of mutants

in our experiments, we chose the second method mentioned above; that is, we applied phage at intervals during the period when we expected that the bacteria had reached the thirteenth division. This method was selected in preference to the other because we were not certain whether the spontaneous-mutation level is always reached at about the thirteenth division or whether this varies with dosage. Moreover, with this method we were able to eliminate possible irregularities in the durations of lag period and division time.

An experiment for determining the total (endpoint) number of mutations induced by irradiation is described below. The assay part of this experiment is so similar to that of the experiment described earlier for determining the number of immediate (zero-point) mutations, that a detailed outline is omitted here and only the results are given. Also, in order to simplify presentation, only the results of treatment with one dose are given, although in every experiment two doses were used.

A 48-hour culture of B/r in M-9, containing 4.1 $\times$ 10$^9$ bacteria per ml., and showing 15 spontaneous mutants per 10$^8$ bacteria, was diluted 25 times in saline. This dilution contained 1.65 $\times$ 10$^8$ bacteria per ml.

Four Petri dishes, each containing 8 cc. of this suspension, were irradiated with a dose of 3000 ergs $\times$ mm.$^{-2}$, which left 10$^4$ survivors per ml.; that is, the rate of survival was 6 $\times$ 10$^{-3}$ %. This suspension was reduced after centrifugation from 30 ml. to 3 ml., giving a suspension containing 1.2 $\times$ 10$^5$ bacteria per ml.

0.1 ml. (or 1.2 $\times$ 10$^4$ bacteria) of the centrifuged suspension was plated on each of 12 agar plates, and incubated. Phage T1 was sprayed on each of four plates after 7, 8, 8$\frac{2}{3}$, and 9 hours. Forty-eight hours later the mutants were counted. The results are given below.

Dose (ergs $\times$ mm.$^{-2}$), 3000.

Number of bacteria per plate, 1.2 $\times$ 10$^4$.

Phage sprayed after 7 hours: Number of mutants per plate,

2-6-2-4; average, 3.5.

Phage sprayed after 8 hours: Number of mutants per plate,

11-8-7; average, 8.7.

Phage sprayed after 8$\frac{2}{3}$ hours: Number of mutants per plate,

31-16-24-18; average, 22.

Phage sprayed after 9 hours: Number of mutants per plate,

19-20-18-22; average, 20.

From these results we see that all the mutants had shown up after 8 hours and 40 minutes. The lag phase for a dose of 3000 ergs was found to be about 3 hours and 30 minutes. Since a division cycle is approximately 20 minutes in duration, 15 divisions had occurred by the time the mutation rate reached a normal level. Therefore, at the time when the phage was applied, each plate had about 1.2

$\times$ 10$^4$ colonies of 3.3 $\times$ 10$^4$ bacteria each—that is, a total of 4 $\times$ 10$^8$ bacteria. As about 0.5 spontaneous mutants appear for each 10$^8$ divisions, we could expect two spontaneous mutants on each plate. The number of induced mutants, therefore, was 22 — 2 = 20 per 1.2 $\times$ 10$^4$ bacteria, or 1670 per 10$^6$ bacteria.

The result of the experiment was that a dose of 3000 ergs $\times$ mm.$^{-2}$ induced 1670 mutants per 10$^6$ surviving bacteria.

## EXPERIMENTAL RESULTS
### Survival Curves

As previously stated (see Technique of Irradiation), the ultraviolet survival curve was used for calculating the dose given to bacteria when the experiments required highly concentrated suspensions. Therefore this curve had to be fairly well known, and was first determined in a special series of ex-
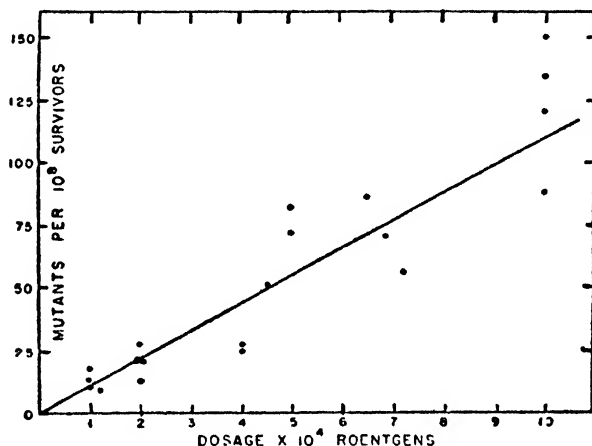


FIG. 3. Zero-point mutants induced by X-rays in B/r. Dots represent experimental results, and circles averages for every tested dosage. The linear plot shows that the mutation rate is proportional to the dosage. The dosage given is that measured at the surface; to obtain the average dose in suspension the correction factor 0.84 should be used.

periments. The full-grown culture was diluted in saline to a concentration of 10$^7$ bacteria per ml., then irradiated in a thin layer, 1 mm. in depth. These conditions avoided any screening of bacteria by one another. The curve was thus determined down to a rate of 10$^{-3}$% survivors. From this rate on, it was necessary to use a more concentrated initial suspension, and the results were not so reliable. (Fig. 1, dotted portion of the curve.)

The ultraviolet survival curve for resting B/r bacteria (Figs. 1 and 4) belongs to a multiple-hit type, conforming closely to the theoretical Poisson 3-hit curve. In semilogarithmic plot it starts with a plateau, followed by a straight line. From a survival rate of about 5 $\times$ 10$^{-3}$ % on, this straight line is broken, the last survivors displaying some slight increase in resistance.

The ultraviolet survival curve for B/r in the growing stage was determined under the same conditions. It shows the same general features, although the growing bacteria are a little more sensitive (Fig. 4); a given dosage leaves from two to three times as many survivors with bacteria in the resting as with bacteria in the growing stage.
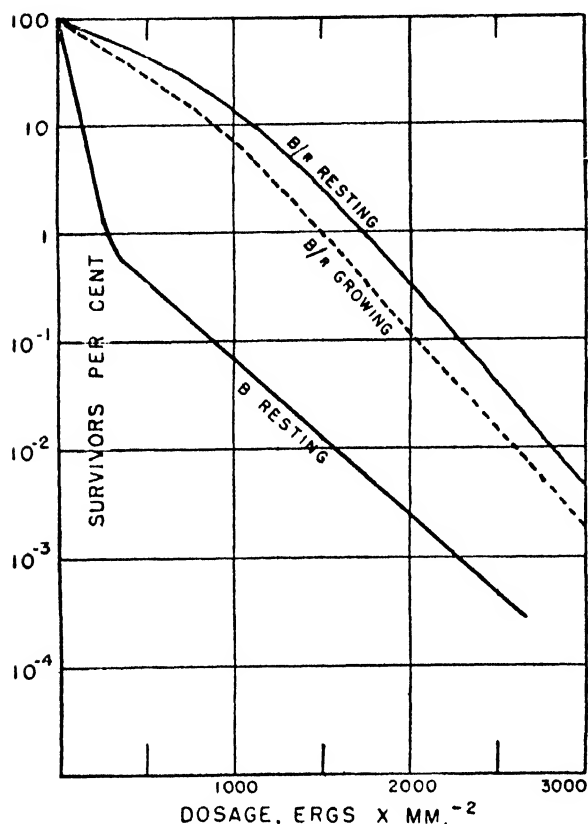


FIG. 4. Ultraviolet (2537 A) survival curves of B/r in the resting and in the growing stage, and of B in the resting stage (semilogarithmic plot). B/r is a little more sensitive in the growing than in the resting stage, and much more resistant than B.

The X-ray survival curve for resting B/r bacteria was obtained from the assays of the mutation experiments themselves. As expected, it was an exponential curve (Fig. 2).

The ultraviolet survival curve for strain B in the resting stage (Fig. 4) was obtained by the same technique. Bacteria of this strain, much more sensitive than B/r, are killed according to an exponential function of the dosage. As has already been observed by Witkin (13), a pronounced change in bacterial sensitivity appears around the survival rate of 1%, as if 1% of the bacteria of the normal strain possess a higher resistance (of the same order as that of B/r) than the other 99%.

Here we wish to draw special attention to the fact

that two bacteria as closely related to each other as B and B/r give ultraviolet survival curves of different types—one exponential, and the other sigmoid—under strictly identical experimental conditions. This makes it seem probable that the sigmoid curves already obtained by others with many bacteria are not necessarily due to a disturbing factor, as clumping or toxic action of the irradiated medium (11), but are the result of typical characteristics of the bacteria themselves.

### Mutations Induced in B/r by Ultraviolet Rays

Fig. 1 shows the results obtained with B/r, using ultraviolet radiation of wave length 2537 A. The dots and circles indicate the individual experimental values, and the curves are drawn to fit them.

The zero-point curve shows that the rate of zero-point mutations increases tremendously with the dosage, going from a value of 20 per $10^8$ for a dose of 1000 ergs $\times$ mm.$^{-2}$ up to a maximum of 70,000 per $10^8$ for a dose four times as great. At this point a plateau is reached, followed by what appears to be a slow drop. The existence of such a plateau agrees with a similar observation by Stadler and Uber (12) concerning mutations (deficiencies) in maize; and Hollaender and Emmons (7), studying mutations induced by ultraviolet in fungus spores, also observed the existence of a maximum rate for a certain dosage. The ascending part of the curve appears complex, rising more and more rapidly with the increases in dosage. It is fairly well represented by the formula, log log $M = k$ D, meaning that the log log of the mutation rate is proportional to the dosage. If one wanted to recognize in this curve the beginning of a multiple-hit curve, then the number of hits assumed would be approximately 10.

The first portion of the end-point curve is similar to that of the zero-point curve; the ratio between end-point and zero-point mutants, in that region of the curves, is about 400:1. Later, the increase in the end-point rate lags behind the increase in the zero-point rate, and the two curves approach each other. The end-point curve does not show any drop, and continues upward until a mutation rate of 2.8% is reached, which is the highest we were able to obtain. The very high dosage used at that point leaves only $2.2 \times 10^{-6}$ % survivors. Such a rate of mutation is high, in view of the fact that we are dealing with very specific mutations.

Examination of Fig. 1 shows that the individual results are somewhat divergent, particularly those relating to zero-point. The different experiments in which the same dose was used sometimes gave results differing from one another by a factor of ten. This is not surprising if one considers how many operations are required to obtain each result. We tried to minimize this divergence by carrying out a large number of experiments. It seems to us that the 65 and 33 results available for the zero-point and end-point mutation rates, respectively, serve to pro-

vide a good picture of the relation between mutation rate and dosage.

### Mutations Induced in B/r by X-rays

In 1943 Croland (2) published results indicating that specific mutations can be induced by X-rays in the bacterium *Moraxella lwoffi*, and that the mutation rate increases with the dosage. Because of a high rate of "killing," the relation between mutation rate and dosage was not determined.

Apparently B/r bacteria are more resistant to X-rays than the strain of Moraxella used by Croland, but still the "killing" rate was so high that we were not able to observe the relation between mutation rate and dosage over such a wide range of dosage for X-rays as for ultraviolet rays. This is why our X-ray curves have comparatively few points—namely, 16 for zero-point and 17 for end-point mutants. From Fig. 3, in which the dots represent experimental results and the circles their averages, it is evident that the relation between zero-point mutants and dosage follows a straight line, which can be considered as the beginning of an exponential one-hit curve. This result agrees with most of the similar studies on dosage-mutation relations made by others, and suggests that mutation is induced by direct action of the radiation on a limited locus of the cell.

TABLE 1. MUTATION RATES (END-POINT) PER $10^6$ BACTERIA INDUCED BY VARIOUS DOSES OF ULTRAVIOLET RADIATION IN GROWING AND RESTING B/r

| Dose (ergs×mm.$^{-2}$) | Growing | Resting |
|---|---|---|
| 1000 | 120 | 120 |
| 2000 | 470 | 500 |
| 3000 | 4400 | 2000 |
| 3500 | 5000 | 5000 |

In Fig. 2, the X-ray data are plotted in such a way that direct comparison can be made with the results of ultraviolet experiments shown in Fig. 1. Examination of Fig. 2 indicates that the end-point rate increases a little more rapidly than the zero-point rate. The ratio between total end-point and zero-point mutation rates varies between 100 and 200.

### Mutation Rate in Growing Bacteria

In our experiments we used primarily resting B/r bacteria. Since it was of interest to know whether the observed results apply specifically to bacteria irradiated in the resting stage or hold true also for bacteria irradiated in the growing stage, a set of experiments was performed with growing bacteria. The results are summarized in Table 1. For comparison, a summary of data obtained with resting bacteria is included in the table.

The data for growing bacteria irradiated with doses of 3000 and 3500 were obtained from one experiment, and the data for doses of 1000 and 2000 represent averages from two experiments. The agreement between the two sets of data appeared to be close enough so that further experiments were not judged necessary.

### Mutation Rate in the B Strain

Experiments on a small scale were made with the B strain, in order to find out whether or not results similar to those of the experiments with B/r would be obtained. The data are presented in Table 2. To facilitate comparison, data for B/r are also included in the table. The data for the two strains agree fairly well.

For the ultraviolet experiments with strain B, the data for zero-point mutants represent averages from ten experiments, those for end-point mutants averages from four experiments; the X-ray data are from one experiment.

These results justify our reasons for choosing the B/r strain for our experiments. Apparently, mutation rate increases with dosage regardless of the "killing" effect of the radiation. Since bacteria of the B/r strain are much more resistant to radiations than bacteria of the B strain, it was possible in our experiments to use higher doses with B/r than with B.

### ANALYSIS OF THE RESULTS
#### Comparison between Induction of Mutations and "Killing"

It has frequently been argued that sterilization of bacteria by radiations, generally designated improperly as "killing," is due to genetic changes in the cells. Moreover, it has been suggested that these changes are probably lethal mutations (11). If this

TABLE 2. SUMMARY OF EXPERIMENTS WITH RESTING BACTERIA OF STRAIN B, AND COMPARABLE DATA FOR STRAIN B/r

| Dose (ergs×mm.$^{-2}$) | Ultraviolet | | | | X-rays | | |
| | Zero-point (per $10^6$) | | End-point (per $10^6$) | | Dose (roentgens) | End-point (per $10^6$) | |
| | B | B/r | B | B/r | | B | B/r |
|---|---|---|---|---|---|---|---|
| 250 | 5 | 7 | 4 | 10 | 40,000 | 13 | 7 |
| 500 | 12 | 10 | 25 | 30 | 60,000 | 40 | 15 |
| 1000 | 20 | 20 | 140 | 120 | | | |
| 2000 | 240 | 180 | 200 | 500 | | | |
| 2500 | 460 | 800 | | | | | |

were so, then it might be expected that some relation would be found between such lethal mutations and specific mutations, such as those we are dealing with, induced by radiations.

Gowen (6), comparing the rates of mutations induced by X-rays in plant viruses, bacteria, and Drosophila, has already pointed out that these rates are of the same order of magnitude, while the rates of inactivation of these organisms by the same radiation differ significantly from one another. Our results, obtained with strains B and B/r (Table 2), clearly show that mutation to phage resistance, induced by either ultraviolet rays or X-rays, increases similarly with increased dosage in both of these bacteria, while sterilization does not—B being much more sensitive than B/r. From this we can infer that when B mutates to B/r the material involved in sterilization by radiation is somewhat changed, while the material involved in mutation from phage-sensitivity to phage-resistance remains the same. In this case, therefore, there is no evidence of any relation between the ability of a radiation to produce sterilization and its ability to produce mutations.

### Quantitative Comparison between Ultraviolet and X-rays

We have seen that, qualitatively, ultraviolet rays and X-rays show similar behavior; that is, both produce zero-point and end-point mutations, and in both cases the rate of mutation increases with the dosage. This increase, however, does not follow the same pattern with ultraviolet as with X-ray treatment, and a comparison of the curves shown in Figs. 1 and 2 may contribute some information about the mechanisms involved in the production of these mutations. (See Discussion.) Still further information may be derived from a comparison of the amounts of energy absorbed by the cell; for these mutations, whatever their mechanism may be, are a result of the absorption of radiant energy. Such comparisons have already been made by several authors, working with other material.

In order to express in terms of the same unit the amounts of ultraviolet and of X-ray energy absorbed by the cell, we must know the absorption coefficient of the latter for both radiations.

For X-rays, where the absorption is distributed at random throughout the whole bacterium, this coefficient depends only upon the wave length. As the flux emitted by our source was very heterogeneous, we are obliged to use for purposes of calculation its average wave length, which is 0.35 A. This transposition would be fallacious if the observed induction of mutations were not independent of the wave length but were produced by a certain fraction of the total radiation. We have not used monochromatic radiations to check this point, but an extensive amount of evidence is available indicating that, within the range of the X-ray spectrum given by our source, the genetic effect is independent of the wave length. As has already been mentioned, this radiation produced, in our experiments, an average of 13,800 ionizations per $\mu^3$ for a dose of $10^4$ roentgens. Considering the volume of B/r as approximately equal to 1 $\mu^3$, and knowing that one ionization corresponds to an average release of energy of 33 eV, or $5.35 \times 10^{-11}$ erg, we can write:

(a) A dose of $10^4$ roentgens corresponds, in our experiments, to $7.4 \times 10^{-7}$ erg absorbed per $\mu^3$ (bacterium).

For the ultraviolet radiation 2537 A, the absorption coefficient of the whole bacterium approximates 3600 cm.⁻¹ (5). This gives an absorption of 30% of the incident radiation by a thickness of 1 $\mu$, which is about the average thickness of a bacterium. We can then write:

(b) A dose of 1000 ergs × mm.⁻² corresponds, in our experiments, to $3 \times 10^{-4}$ erg absorbed per $\mu^3$ (bacterium).

But here a difficulty appears, owing to the selective absorption of ultraviolet radiation. Inside the bacterium the absorbed energy is unequally distributed, with a high maximum in the nucleoproteins, where the density of absorbed energy can reach ten times the value indicated above.

With these reservations in mind, we can proceed, on the basis of estimates (a) and (b), to a quantitative comparison of the results obtained. This comparison is summarized in Table 3, which includes the entire range of X-ray doses used and the ultraviolet doses corresponding to the ascending part of the zero-point curve. A quantitative comparison is given here of doses, amounts of energy absorbed per bacterium, percentages of survivors, and numbers of mutants.

Analysis of the data presented in Table 3 throws light on the following problems:

*"Killing."* X-rays suppress bacterial division with

TABLE 3. COMPARISON OF ULTRAVIOLET AND X-RAYS IN PRODUCTION OF MUTATIONS IN RESTING B/r

| Dose | | Absorbed energy per bacterium (ergs) | | Survivors (%) | Zero-point mutations per $10^8$ | | End-point mutations per $10^6$ | |
|---|---|---|---|---|---|---|---|---|
| Ultraviolet (ergs×mm.⁻²) | X-rays (roentgens) | Ultraviolet | X-rays | | Ultraviolet | X-rays | Ultraviolet | X-rays |
| 1130 | 20,000 | $3.4\times10^{-4}$ | $1.48\times10^{-6}$ | 10 | 22 | 22 | 180 | 28 |
| 1750 | 40,000 | $5.2\times10^{-4}$ | $2.96\times10^{-6}$ | 1 | 90 | 44 | 420 | 80 |
| 2300 | 60,000 | $6.9\times10^{-4}$ | $4.44\times10^{-6}$ | $10^{-1}$ | 400 | 66 | 800 | 140 |
| 2850 | 80,000 | $8.5\times10^{-4}$ | $5.92\times10^{-6}$ | $10^{-2}$ | 3000 | 88 | 1400 | 180 |
| 3550 | 100,000 | $10.8\times10^{-4}$ | $7.40\times10^{-6}$ | $10^{-3}$ | 45,000 | 110 | 3300 | 200 |

greater efficiency than do ultraviolet rays, inasmuch as they give the same rate of survival with the absorption of about 200 times less energy by the cell. If the mechanism involved in "killing" is the same with both radiations, it can be said that this mechanism has about 200 more chances of being started by one ionization than by the few ultraviolet photons needed (probably 3, according to the shape of the survival curve in Fig. 1). This value agrees with that already obtained in experiments with a paradysenteric bacillus (*9*), where this ratio was found to be 215:1. In respect to a lethal action, such as sterilization, the "yield" of the ionization is much higher than that of the ultraviolet photon.

*Relation between "killing" and mutation.* The absence of correlation between "killing" and production of mutations, already observed in experiments where the same radiation was used on two different strains of bacteria (B and B/r), is found again here, where two different radiations were used on the same bacterium (B/r).

*Mutations.* According to results already obtained by other authors, X-rays appear to be more efficient than ultraviolet rays in producing mutations. Our results might seem to lead to the opposite conclusion, since we found that ultraviolet rays gave up to nearly 3 mutations per 100, while with X-rays we were unable to obtain more than 2 mutations per $10^4$. But the first prerequisite for the detection of a bacterial mutation is survival and division of the mutant. It is not surprising that the violent action of an ionization, with its large quantum, is more efficient in producing lethal effects than the less violent action of an ultraviolet photon. On the other hand, we can assume that the latter has a greater chance of producing a mutation without killing the cell. This may explain, in our experiments, the apparent failure of X-rays; and for a clear picture we must compare the results on the basis of equal amounts of absorbed energy.

Let us first consider zero-point mutations. An absorbed ultraviolet energy of $8.5 \times 10^{-4}$ erg per cell, for example, produces 3000 mutations per $10^8$ survivors (Table 3). With X-rays, according to the exponential relation seen in Fig. 3 between mutation rate and dosage, the same rate of 3000 mutations per $10^8$ survivors would be obtained with a dose of $2.7 \times 10^6$ roentgens—that is, an absorbed energy of $2.0 \times 10^{-4}$ erg per cell. This energy is close to, and even smaller than, the ultraviolet value. But with this X-ray dose we would expect to obtain about one survivor per $10^{100}$ bacteria, and therefore such a high mutation rate can never be observed with X-rays.

Now, in the case of end-point mutations, the aforementioned ultraviolet dose of $8.5 \times 10^{-4}$ erg absorbed per cell produces 1400 mutations per $10^6$ survivors. From the X-ray end-point curve of Fig. 2, one could expect the same rate to be reached with a dose of about $1.5 \times 10^6$ roentgens—that is, an absorbed energy of $1.1 \times 10^{-4}$ erg per cell. In the present instance, X-rays would produce the same rate of mutation as ultraviolet rays with nearly ten times less absorbed energy. This value of 10 represents a lower limit, for, as previously mentioned, the absorption of ultraviolet in genetic parts of the cell is higher than the average values of absorption within the whole cell. Therefore, our results agree with the general conclusions of others, showing that X-rays are more efficient than ultraviolet rays in producing mutations.

## DISCUSSION

Using bacterial mutation resulting in resistance to a bacteriophage as a criterion, it is possible to test the genetic effect of radiations immediately following treatment and also after the treated cells have been allowed to divide. With this technique, two types of effect were observed: one immediate, producing phenotypic changes in the treated cells, and the other delayed, responsible for phenotypic changes appearing in the offspring of these cells.

Before entering on a discussion of interpretations of the observed results, we will briefly review the pertinent features concerning the relation between bacteria and phages. It is known from the work of Delbrück (*3*), that one sensitive bacterium may adsorb as many as 250 phage particles, while resistant bacteria do not adsorb any. Presumably, one or several adsorbed particles enter a bacterium and multiply therein until finally the bacterium is lysed —it bursts and liberates phage particles. There are at least two critical steps, therefore, in the life of a bacterium that determine whether or not it will be lysed by a phage, if a phage is present. These steps are the adsorption of phage by the surface of the bacterium and the multiplication of phage within it. It is evident that if either of these two processes is prevented from occurring the bacterium will be resistant to the phage; and it is probable that if a change in a bacterium is capable of making it resistant it must be capable of blocking one of these two processes. If the change is to be detected, however, it is necessary that the condition responsible for resistance be transmitted to the progeny of the affected bacterium—i.e., that it become an inherited property. Our scoring of mutants is made by colony counts, and by the time this is done the original resistant bacterium has multiplied to form several million bacteria—all of them resistant, since the multiplication takes place in contact with phage.

The condition responsible for nonadsorption of the phage may be produced either by a direct effect on the surface of the bacterium, or by a change within the bacterium affecting the forces responsible for adsorption. Since, presumably, the surface of a bacterium has a fairly large number of points at which a phage particle might be adsorbed (receptors), it is difficult to conceive how each of these individually could be affected by radiation in such a way as to give the results observed in our experi-

ments. The data obtained from the experiments with ultraviolet radiation (Fig. 1) indicate a complex reaction, probably requiring several hits to produce a mutant; but even so it is unlikely that one hit would be provided for every receptor, and the data obtained from the experiments with X-rays indicate that one ionization is sufficient to produce a mutant. It is true that it may not be necessary to assume that a direct hit for each receptor is essential to produce changes in all the receptors. It is possible, by assuming some sort of chain reaction, to postulate a situation in which the number of hits indicated by our results would be adequate to affect the whole surface of a bacterium; and a similar assumption would account for the observed behavior of X-rays. For instance, an electric change in some part of the membrane could lead to a new general equilibrium of the latter, with new characteristics of adsorption and of permeability. However, we would still have to explain the mechanism by which resistance is transmitted to the progeny of the affected bacterium. If the resistance were caused by an effect of radiation on the membrane, and the induced characteristic were transmitted to the offspring, this would mean that in bacteria the membrane, or regions thereof, have genetic individuality, such as is generally attributed to genes and chromosomes. We feel that such an assumption would be justified only if no other explanation were possible, which, in this particular instance, is not the case.

Additional evidence against the assumption that resistant mutants are produced by direct action of a radiation on the membrane of a bacterium is contributed by the observed effects of X-rays and ultraviolet rays on killing and on mutation rate. It has been shown earlier that different sites (materials) of the bacterial cell are affected in the production of killing and of mutation by radiations, and that ultraviolet rays are more efficient than X-rays in producing mutations without killing the cell. If mutations were induced by action on the bacterial membrane, and killing by action within the bacterium, such a difference between the effects of the two radiations would hardly be expected. On the other hand, if the sites of the two effects—i.e., killing and mutation—are close together, an ionization, whose high energy (33 eV) sometimes diffuses, could affect both of them together, while an ultraviolet photon (5 eV), which is absorbed by a single molecule, could not.

It is entirely possible that the surface of a bacterium may be affected indirectly; i.e., that a change occurring within the bacterium may, through its effect on the surface, cause it to lose the ability to adsorb phage particles. Under the conditions of our experiments, an internal change affecting adsorption cannot be distinguished from an internal change affecting multiplication of the phage. Therefore we shall not make any attempt to differentiate between these two possibilities.

We shall review here the interpretations discussed by Demerec (4), and see whether the additional evidence makes any of them more probable.

One of the simplest explanations of the appearance of mutants both immediately after treatment and after several bacterial divisions is the assumption that the bacteria in question are diploid, and that the immediate mutants are the result of coincidental changes of both alleles by two independent hits, while the delayed mutants are the result of segregation of heterozygotes. There were two objections to this explanation: (1) that the proportion of immediate mutants is larger than would be expected if they were due to coincidental changes; and (2) that the majority of mutants occur during late bacterial generations, rather than during the early generations as would be expected if delayed mutants were due to simple segregation. Our new data have not eliminated either of these objections.

Another possible explanation of the observed delay in the appearance of some of the mutants supposes that the bacterial population used in experiments may not be homogeneous; that is, that some are haploid, some diploid, and some polyploid. By assuming certain proportions of the various types, a mixture could be postulated that would give the observed results. The additional data from our experiments with growing and resting bacteria do not confirm this assumption. We observed no difference in mutation rate between resting and growing bacteria (Table 1), while such a difference could be expected if delayed appearance of mutants were due to polyploidy, since it is probable that the proportions of haploid, diploid, and polyploid individuals would differ in growing and in resting bacteria.

A third possible assumption is that all mutations occur during irradiation by action on a gene, but that some are delayed in their manifestation until the supply of substrate manufactured by the gene and necessary for production of the material that makes the bacterium sensitive to a phage is exhausted. Our experiments have not added new information regarding this possibility. A detailed study of the appearance of delayed mutants during each bacterial generation should throw some light on the problem.

Finally, still another hypothesis may be considered to account for the results obtained. It may be assumed that two types of change are induced by radiation: one type affecting the gene, and the other affecting either the chromosomes or the cytoplasm in such a way as to increase the mutability of the gene system. The latter type of change would decrease in effectiveness with each cell division and, after a number of divisions, lose its potency. Our present experiments contribute no new evidence either for or against this hypothesis.

In conclusion it may be stated that the evidence available at present does not permit a definite judgment regarding the mechanism of immediate and

delayed mutations induced by radiations. All we are justified in saying is that the probable site of change is inside the bacterium; that the change is complex, requiring as a primary step the absorption of radiant energy by several molecules; and that the volume within which the change occurs is not larger than might be affected by a single cluster of ionizations. This change is transmitted to the progeny; hence it behaves as a mutation. Since there is no valid reason to assume that bacteria do not have genes and chromosomes, the most probable explanation of the mechanism of the observed behavior is that it is a gene mutation, although this does not exclude the possibility that in addition to the gene some other material may be affected. The complexity of the change, suggested by the results obtained with ultraviolet radiation, could be explained by assuming that the gene is represented several times, a condition that would exist if the chromosome consisted of a number of chromonemata.

## ACKNOWLEDGMENTS

## SUMMARY

Ultraviolet rays (2537 A) and X-rays induce mutations from sensitivity to resistance to bacteriophage T1 in the B/r and B strains of *E. coli*.

Some of these mutations express themselves before the treated bacteria pass through the first division (zero-point mutations), while others show up during subsequent divisions. All detectable induced mutations have been expressed after about 13 bacterial generations (end-point mutations).

The relation between mutation rate and dosage was determined for the zero point and the end point for both radiations. The results shown in Fig. 1 and Fig. 2 indicate a complex situation for ultraviolet radiation and a simpler one for X-radiation.

Similar results were obtained with resting and with growing bacteria, and with B/r and with B strains, which differ in their resistance to radiations.

The results show no evidence of any relation between the ability of a radiation to produce sterilization and its ability to produce mutations.

The highest rate of mutation, 2.8 per 100 survivors, was obtained after ultraviolet treatment with 6500 ergs × mm.$^{-2}$.

When similar amounts of energy are absorbed by the cell, either from ultraviolet or from X-radiation, similar rates of mutation are observed; while the sterilization rate is considerably higher with X-rays than with ultraviolet.

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## DISCUSSION

PONTECORVO: I would like to call attention to Auerbach and Robson's work on chemically induced genetical changes, since it may have a bearing on both the "delayed effect" observed by Demerec and Latarjet in bacterial colonies and the qualitative results of Tatum and Ryan. This work was started about 1941, and most of the results were already available about one year later; but for security reasons publication has been delayed until very recently. Most of the work is now in press (Proc. Roy. Soc. Edin., B). A series of chemicals, the most effective of which is mustard gas, have been tested, and a theory of their physicochemical action in producing mutations has been worked out (mainly by J. G. Carr). The theory predicts a range of mutagenic activity in a large number of substances, and preliminary tests to verify the theory have been successful. As for the effects on chromosomes and genes, S- and N-mustards and other chemicals have been shown in both Drosophila and plant material to behave qualitatively like X-rays; i.e., they produce all kinds of mutations and rearrangements (the salivary-gland cytology of rearrangements has been

carried out by the Slizynskis). However, they differ from X-rays in some important respects: (1) for the same frequency of mutations there is a considerable shortage of translocations; (2) many of the mutations after treating sperms arise in mosaic form; (3) there seems to be a delayed effect, certainly not due to lingering of the chemical, in the sense that mutations can arise very many cell generations after treatment. I am sorry I can't give fuller details because I am just quoting from memory, but the work has been done on such a large scale and with such analytical detail that it would be necessary for all those embarking on the study of chemically induced genetical changes to become thoroughly acquainted with it as soon as possible.

# COMPLEX REPRODUCTIVE PROCESSES
# IN BACTERIA

## L. DIENES

### Observations on Streptobacillus moniliformis and Various other Gram-Negative Bacteria

The question of whether bacteria multiply exclusively by binary fission, or whether, in addition, they have more complex reproductive processes, has more than academic interest. Binary fission makes possible the segregation of hereditary characteristics, but it offers no means for new combinations and rearrangements of them. It is difficult to believe that in bacteria no mechanism exists by which the properties acquired by variants can be incorporated into the species and by which new combinations of hereditary characteristics may be produced which are advantageous in a given situation. In the case of yeasts and rust fungi, the importance of sexual processes for adaptation to new conditions is apparent from the observations discussed previously in this Symposium.

The idea that complex reproductive processes exist in bacteria has persisted throughout the whole history of bacteriology. Lohnis (13) in a review published in 1921 collected many observations, even from the earliest period of bacteriology, suggesting such processes. Klieneberger (10) reviewed in 1930 the observations that were most suggestive in this respect and also studied some of them experimentally. Many authors have claimed to have observed filterable forms in bacterial cultures. It is fair to say, without trying to assess in detail the value of these observations, that they remained only suggestive and that no instance of a complex reproductive process which could be regularly reproduced and studied in detail was observed in bacteria.

A discovery made by Klieneberger (11) twelve years ago opened a new approach to this problem. She noticed that in cultures of *Streptobacillus moniliformis* tiny colonies developed, which were different in appearance from the rest of the culture and consisted of organisms with different morphology. The organisms in these tiny colonies, which Klieneberger called $L_1$, were different from anything we had been accustomed to see in bacterial cultures. They were similar to the Pleuropneumonia group of organisms, which some authors put in a class different from bacteria. The organisms in the $L_1$ colonies, as in the colonies of the Pleuropneumonia group, are very soft and fragile, and in a certain phase of their life are small enough to pass through bacterial filters. They tend to swell up into large, round forms from 3 to 10 micra in diameter, which in turn reproduce the small forms.

This reproductive process is also characteristic of the Pleuropneumonia group. Klieneberger explained the presence of these small colonies in the culture as a phenomenon of symbiosis between two organisms not related genetically and not belonging in the same class. She still adheres to this explanation (12). The $L_1$ has been studied by various authors (3, 2, 9, 1, 17, 18). All have come to the conclusion that it is a growth form of the bacillus. This opinion is based on the following observations. (1) The morphology of $L_1$, although it is unusual, is distinctly bacillary. The small forms of $L_1$, as well as those of the whole Pleuropneumonia group, are small bacilli or fine bacillary filaments, which have a tendency to become round and also to swell up into large, round bodies. A similar tendency has occasionally been observed in common bacterial species such as *B. coli* and *H. influenzae*. The general morphology of $L_1$ is unusual, but its cultures do not contain anything with which we are not familiar in other pleomorphic bacterial cultures; and on a morphological basis there is no reason to separate the $L_1$ and the whole Pleuropneumonia group from bacteria. (2) The derivation of $L_1$ from the Streptobacillus can be directly observed. It occurs in the following way: the bacilli swell up into large, round bodies, which, if appropriately transplanted, germinate and give rise to the $L_1$. The whole process, the swelling of bacteria and their germination, can be observed directly under the microscope. (3) The $L_1$ is serologically similar to the Streptobacillus. Klieneberger observed some difference between the two forms in agglutinin absorption experiments, but this difference was minimal and may have been due to the fact that the $L_1$ was not used in sufficient quantity. (4) For a variable period of time following isolation, occasionally many months, the $L_1$ will return to the usual bacterial form if it is transplanted to broth. (5) Ordinary bacilli are reproduced in some strains of *Streptobacillus moniliformis* inside the large bodies formed by the bacilli (6). This observation suggests that the $L_1$ is an intermediary phase in the reproductive process leading from bacilli to large bodies and back again to the bacilli.

Klieneberger makes various suppositions to bring these observations into agreement with her idea that the $L_1$ and *S. moniliformis* are different organisms. According to her, the large bodies in the cultures of *S. moniliformis* do not develop from swelling of the bacilli, as occurs in many other species, but are parasites enveloping the bacilli. The small

forms of these parasites, according to her, are always present in cultures of *S. moniliformis,* and are visible in large numbers in autolysed cultures. Both suppositions are contrary to the direct observation of the development of the cultures, and there is no real evidence in support of them. When the development of the bacillus in agar transplants is studied, it is apparent that the $L_1$ starts to grow from the transplanted large bodies and that the granules visible in autolysed cultures are not viable and never grow into $L_1$ colonies. Similar observations have been made in other bacterial species such as Bacteroides, *B. coli,* and *H. influenzae.* The L-type organisms are not present in the bacterial cultures in visible or culturable form, except when the cultures are mixed and contain both types of colonies, and they grow exclusively from the large bodies produced by the swelling of bacteria. Klieneberger explains the serological similarity between $L_1$ and Streptobacillus by the supposition that the $L_1$ is always present in large numbers in the bacterial culture. As was just mentioned, this supposition is contrary to actual observation. On the other hand she explains the recovery of the bacillus from the $L_1$ cultures by the supposition that bacilli are included in and are carried by the $L_1$ colonies. There is a marked difference between the morphology of

the bacilli and of the $L_1$, and if bacilli were present and grew in every L colony, they would be apparent in appropriately stained preparations. With an agar staining technique which will be described later, every organism in young colonies is clearly visible. Bacilli corresponding to the Streptobacillus are never visible in $L_1$ colonies. It is admittedly difficult to recognize contamination of a culture with a morphologically similar organism—as, for instance, the contamination of one strain of Clostridium with another—but the Streptobacillus is not only morphologically different from the $L_1$, it is a larger organism and grows much faster. Brown and Nunemaker (*1*) carried an $L_1$ strain through 120 transfers on solid medium, picking individual colonies, without a change in its ability to return to bacillary form. It is difficult to believe that the bacillus was carried as a contaminant of every colony of $L_1$ and never grew in recognizable form.

It seemed advisable to discuss at length the opinion of Klieneberger, who discovered the $L_1$ and made important contributions to its study; but the arguments with which she supports her theoretical conclusions are not convincing. According to the evidence available at present, the change of the Streptobacillus into the $L_1$ and the return of the $L_1$ to the bacillary form is the first easily repro-

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### EXPLANATION OF FIGURES 1-13 (see opposite page)

FIGS. 1-5 and 7-11 illustrate the observations made on *Streptobacillus moniliformis* and $L_1$, Figs. 6 and 12-21 those made on a strain of Bacteroides (132), and Figs. 22-30 those made on Proteus. As far as possible, the photographs were made from wet preparations. A comparison of the large bodies of Bacteroides in Fig. 16, which was made from a wet preparation, with the large bodies in Fig. 27, made from a dry preparation, shows the advantage of the former. The large bodies of Proteus in a wet preparation are similar in every respect to those of Bacteroides. In some cases lack of time prevented the use of wet preparations, which have to be prepared just prior to photography, since they are not permanent. It was necessary to use dried stained-agar preparations in photographing L-type colonies, because the growth extending into the agar cannot be focused in wet preparations simultaneously with the structures on the surface of the agar. Dried preparations were made by placing a thin slice of agar containing the culture on a stained coverslip and allowing it to dry. The colonies are vertically compressed in such preparations, and a larger portion of the colony can be brought into focus. In Figs. 17 and 18, the germination of the large body into L type of growth is presented as it is visible in wet preparations, 17 representing the surface of the agar, 18 a level below the surface. The magnification of the majority of the photographs is × 2000 or × 3000. By the use of this high magnification the details should remain visible in the printed reproductions. For the same reason photographs 8 and 9 were still further enlarged to × 4500.

FIGS. 1 and 2 show the edge of a colony of *Streptobacillus moniliformis* in wet and dry agar preparations respectively (× 3000). The consecutive stages in the swelling of the filaments and the transformation into large bodies are apparent.

FIGS. 3 and 5 (× 3000) represent a transplant of the culture shown in Fig. 2 after 12 hours of incubation. The bacterial filaments have not multiplied noticeably during this time and have not changed in appearance. Several large bodies have increased considerably in size, and small soft granules, which invade the agar and form darkly stained aggregates, have grown out of them. The large bodies from which L-type growth started are marked with an arrow.

FIGS. 4 and 10 illustrate young $L_1$ colonies as they appear in dry and in wet preparations. In the latter only the surface of the colony is apparent, the rest is embedded in the medium. It is apparent that the colonies consist of small forms whose exact shape is not visible because they are in clumps and are not on a plane surface.

FIG. 6 shows in unstained preparation the germination of a large body of a Bacteroides strain. The contour of the large body is more clearly visible than in the preceding dry preparation, but the morphology of L-type growth is less apparent.

FIGS. 7, 8, and 9 illustrate the development of bacteria in the large bodies (7, × 3000; 8 and 9, × 4500). Photographs 7 and 9 show a loose, filamentous growth produced by growth of the transplanted bacillary filaments, while the large bodies appear to be densely filled with bacilli. In photograph 8 two large bodies are apparent; one shows no inner structure, the other is completely filled with bacillary forms. In the course of further growth the bacilli developing in the large bodies become similar to those developing from bacterial filaments.

FIG. 11 shows with low magnification (× 200) fully developed $L_1$ colonies. The dense center embedded in the agar and the lightly stained periphery on the surface consisting of large round bodies are characteristic of these colonies.

FIGS. 12 (× 3000) and 13 (× 500) illustrate a young and a well-developed L type of colony of Bacteroides strain 132. The similarity of these colonies to the $L_1$ isolated from *Streptobacillus moniliformis* is apparent.
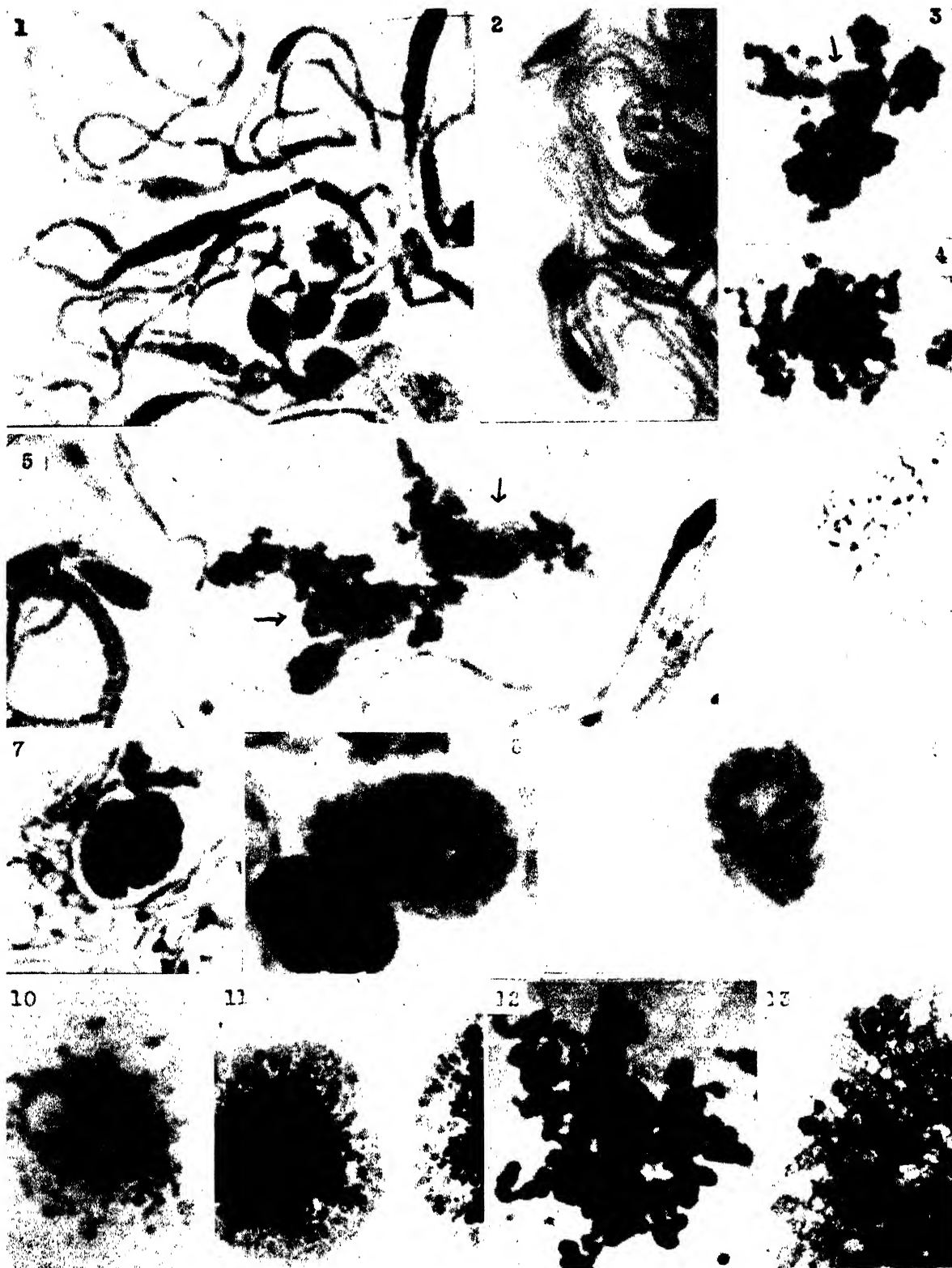
FIGS. 1-13 (see opposite page for legends).
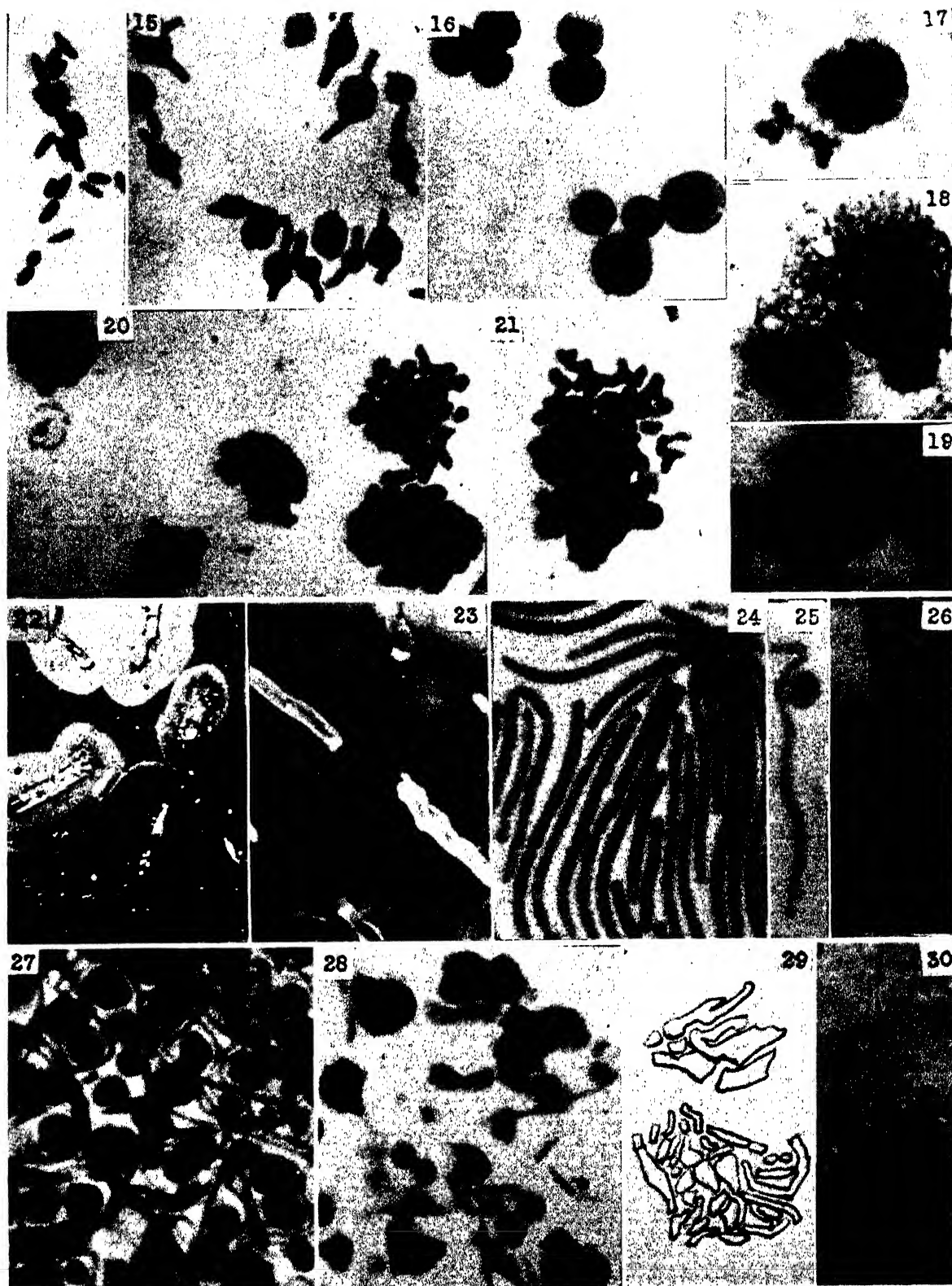
FIGS. 14-30 (see opposite page for legends).

ducible case of a life cycle in bacteria including forms with markedly different morphology and reproductive processes.

The significance of these profound changes in the bacterium is not known. There is only one process of which we know the significance to which the formation of the $L_1$ presents similarities. This is the germination of the single spores from the ascospores of the yeast. This process was described by Winge (*19*), who gave the name "haploform" to the descendants of the single spores. Colonies of the $L_1$ among colonies of the Streptobacillus have an appearance similar to the tiny, rough, haploform yeast colonies among the usual yeast colonies. The comparative morphology of the $L_1$ and of the haploform yeast in relation to their parent organisms is quite similar. Both are relatively small and have a tendency to produce round forms and swell to a larger size. The colonies in both cases often die out; but if they become well adapted to continuous growth, they usually lose their ability to return to the parent forms. The most important function of the haploform yeast, conjugation, has not been observed in $L_1$, but the similarities are marked enough so that we should take notice of them. Heilman (*9*) expressed the opinion that the $L_1$ is a degraded form of the bacillus. This designation would fit haploform yeasts as well, if their sexual functions were not known. It is unlikely that such a profound change as transformation into $L_1$ has no significance, and its discovery puts a problem of great interest before us.

Observations similar to those made with Streptobacillus were made with slight variations in Bacteroides strains (*8*). The bacteria or bacterial filaments in the cultures of certain strains of this species swell up into large bodies, and these reproduce either a growth similar to $L_1$ or a growth of the usual bacilli. The swelling of the bacilli into large bodies, the germination of these into L-type forms, and the reproduction of bacilli from the large bodies was observed directly in slide cultures. Photographs of this consecutive process have been published (*8*). The morphology of these processes can be observed more easily in Bacteroides than in Streptobacillus because the organisms are larger and because very often all the bacteria in the culture are transformed into large bodies and thus can be studied in the absence of usual bacillary forms. The L form can be kept indefinitely in cultivation, but its return to bacilli has not been observed in this species and its serological properties have not been studied.

Besides these two species, the development of large bodies into both L-type and bacillary colonies has been observed in *B. proteus, E. coli*, and *H. influenzae* (*5*). Development of L-type colonies alone was observed in a Flavobacterium (*5*), in *H. parainfluenzae* (*7*), and in *N. gonorrheae* (*4*). In all of these species, the similarity to the analogous processes in *S. moniliformis*, manifested by the formation of the large bodies and their devel-

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EXPLANATION OF FIGURES 14-30 (see opposite page)

Figs. 14, 15, and 16 ($\times$ 2000) show the development of large bodies from bacilli in broth culture of the Bacteroides strain. Photograph 14 shows the organism after 4 hours, photograph 15 after 9 hours, and photograph 16 after 17 hours of incubation. It is apparent in photograph 15 that almost all bacilli develop a swelling at about the same time. The swelling is usually double and involves two segments of the filament. After 17 hours the culture consists exclusively of large round bodies.

Figs. 20 and 21 ($\times$ 2000) show the appearance of these large bodies after having been transferred to agar and incubated for six hours. The large body in photograph 21 has increased considerably in size and, like the others, is breaking up into bacteria.

Figs. 6, 17, 18 and 19 show the development of L-type colonies from the large bodies. In photograph 6, which was made of an unstained preparation, the large body has increased in size, it is flat, and on one side there is a granular mass growing into the agar. This is the developing L-type colony. Fig. 17 shows this process in a dry stained-agar preparation. The large body is somewhat increased in size, and L-type growth has begun to grow out of it. Figs. 18 and 19 show the development of $L_1$ from the large body in a stained wet preparation. Fig. 18 shows the large body on the surface of the agar. It is considerably increased in size, deformed, and filled with granules. At the lower right of the body is the shadow of a dark mass corresponding to the L type of growth situated under the surface. Photograph 19 is focused on this dark mass. The large body is not visible, but parts of the L-type growth are apparent.

Fig. 22 shows the development of spreading halos around Proteus cultures (natural size). Fig. 23 shows a similar plate after 24 hours of incubation. In the latter, the areas covered by different strains are apparently separated by lines. In the upper part of the photograph two halos belonging to the same strain merge without hindrance.

Fig. 24 shows the long filaments, which produce the halo by moving on the surface of the agar.

In Fig. 27, showing the contact zone of the two halos, almost all filaments are transformed into large bodies. The photograph was made of a dried preparation, and the large bodies are not so clearly defined as in wet preparations.

In Figs. 25 and 26, made from wet preparations, filaments are shown with fairly large as well as small round bodies attached to them.

Fig. 28 represents transplanted large bodies after approximately an hour's incubation. Some of the large bodies have increased in size and have become deformed.

Fig. 29 shows two drawings of tiny colonies formed by the disintegration of a large body after one and two hours of incubation respectively.

Fig. 30 shows a tiny L-type colony growing out of a large body, which was produced in tap water and which was slightly deformed when transplanted.

opment into both L-type and bacterial colonies, indicates that they all represent the same fundamental process. This process is apparently widely distributed among Gram-negative bacilli. In addition it occurs in *N. gonorrheae.* Transformation into large bodies under normal conditions of cultivation has been observed in Streptococci and Staphylococci, in large spore-bearing aerobic bacilli, and in many other unidentified species. Although further development of the large bodies was not observed in these instances, the occurrence in these cultures of large bodies indicates that the reproductive processes observed in Gram-negative bacilli are probably a general characteristic of all bacteria.

Figs. 1-30 illustrate the development of large bodies from bacilli and the subsequent production of bacilli and L-type colonies from these large bodies in *S. moniliformis,* Bacteroides, and *B. proteus.* The photographs and the explanatory notes will give a more accurate impression of the actual observations than would be possible by detailed description.

The absence of detailed and well-documented observations in the literature concerning the further development of large bodies is due to the fact that this phenomenon is usually observed accidentally and cannot be reproduced at will. Under normal conditions of culture, large bodies are produced in sufficient numbers for study only in a few species of bacteria and in a few exceptional strains of other species. As a rule they are produced for only a short period of time after original isolation. The success of the author in observing these peculiar reproductive processes in many species was made possible by the study of large numbers of cultures obtained directly from pathological lesions and by the storage of such cultures in carbon-dioxide ice. The study of large bodies has been misdirected by the observation that such bodies are produced in many species by slightly toxic influences, such as the presence of Ca, Li, Cd, and Hg salts, penicillin, and some other substances in the media. These easily available, artificially induced large bodies have been studied extensively, while the naturally occurring large bodies have received little attention. The experience of the author corresponds to that of other investigators in the observation that the large bodies produced by toxic influences never germinate and reproduce. They are similar in appearance to the large bodies that develop in ordinary cultures, but do not have a similar potentiality for development.

An occasional large body is produced under normal conditions of growth in almost all species. Transformation of the majority of the organisms of a culture into large bodies, however, is in most species exceptional. There are a few species, such as *Streptobacillus moniliformis* and *Bacteroides funduliformis,* in which transformation into large bodies is apparently the rule, and this process is

used as a characteristic for the definition of these species. This use is probably an error, because even in these species pleomorphism is variable, and in their natural habitats—i.e., in the pharynx of rats and in the human colon—there are similar organisms that are not pleomorphic. These are indistinguishable from the pleomorphic organisms after the latter lose their pleomorphism. *S. moniliformis* and *B. funduliformis* are probably essentially similar to the pleomorphic strains of other species; and transformation into large bodies indicates an exceptional condition, which occurs more often and is retained longer in these two species than in others.

Abundant formation of large bodies under the usual conditions of cultivation is a property of the strain and is not necessarily caused by peculiarities of the medium. For example, a colon bacillus strain (1706), described previously (5), developed into large bodies on Endo's medium, blood agar, ascitic agar, and broth. This property was lost, however, after a few passages through broth. The influence of the medium is usually apparent in that in a richer medium, which permits more abundant growth, the pleomorphism develops earlier and is more pronounced. The strains possessing a pronounced tendency to produce large bodies also differ in other respects from the usual strains; their most remarkable characteristics are variability and a tendency to autolysis. In the colon bacillus culture mentioned above, small colonies containing large bodies, rough colonies consisting of long filaments, smooth colonies, and mucoid colonies sometimes developed simultaneously. The colonies that formed large bodies died out within a short time (24 to 48 hours) or gave only a few colonies in transplants. A further characteristic of the pleomorphic strains, in all species in which they have been observed, is that each strain presents a definite individuality, which persists throughout the period during which pleomorphism is present. Every strain of *S. moniliformis* or of pleomorphic colon bacillus or of Bacteroides has its characteristic pattern of transformation into large bodies, of germination of these into pleuropneumonia-like or bacterial colonies, of variability, and of autolysis. The author studied extensively six strains of *S. moniliformis.* He was able to identify these strains without difficulty by morphological appearance and, in several cases, the $L_1$ isolated from them as well. This marked individuality of the strains is an important argument against the supposition that the complex reproductive processes observed in them are degenerative. One obtains the impression from the study of these strains that the bacteria pass through periods of increased variability connected with the appearance of an unusual reproductive process. Strains presenting these phenomena were in all instances pathogenic species cultivated from pathological material. The period of variability persists

for varying lengths of time in different strains— some, like *S. moniliformis*, retain it for a long time while others lose it after a few transplants. Once this property had been lost, the author was never able to restore it by changing the medium or by animal passage.

It is probable that transformation into large bodies is induced by a secretion or by a metabolic product of bacteria. Often the majority of the bacteria are involved at the same time in broth cultures or on extensive areas of agar plates, usually at the center of the inoculated portion. In a strain of Bacteroides in broth culture, for instance, a rapid multiplication in the form of short filaments occurred for 8 to 12 hours. Almost all filaments began to show a swelling at their center at about the same time. During the following hours all were transformed into large, round bodies and further multiplication stopped. Only a few bacteria retained their regular form, and when observed in slide cultures they were found not to be viable. If at any time during this process the swollen bacteria were transferred to fresh medium, they disintegrated into small bacilli and continued for a certain period to multiply in such form. It is apparent that something, presumably produced by the bacteria, was present in the medium and induced the transformation. A similar transformation en masse into large bodies precedes the autolysis of many freshly isolated strains of *H. influenzae*, *B. coli*, and *N. gonorrheae*.

The supposition that the transformation into large bodies depends on substances diffusing through the medium is further supported by the observation previously mentioned that toxic substances, including penicillin, often induce the transformation of bacteria into large bodies. These latter large bodies, however, never show reproductive processes, and for this reason they are generally regarded as degenerative. It has been supposed that their formation is due to the fact that the bacteria under adverse conditions retain the ability to grow longer than the ability to divide. As similar large forms produced under normal conditions of growth are viable and are in all probability part of a complex reproductive process, it is more likely that the toxic substances start this reproductive process but are not able to bring it to its natural end. The observation that the bacteria swell into large bodies in toxic media makes it more difficult to accept the hypothesis that the large bodies are special reproductive forms when they are produced under normal conditions of growth. However, observations made under normal conditions have a preponderant significance compared with observations relating to the effect of toxic substances.

The observations that have been reviewed refer to the derivation of various morphological forms from other dissimilar forms. Some of these forms are very fragile or very small; some are situated under the surface of the medium. To give an impression of the reliability of the observations, it is necessary to indicate the methods by which they were obtained. Only methods that show the organisms on the media on which they were grown, in an undistorted form and in their original relationship to one another, and which show every organism present in the culture, will give reliable results. The usual smear and impression preparations are not satisfactory. Study of the broth cultures is rarely sufficient, because the organisms do not remain in their natural connections and their derivation from other forms usually cannot be established. Examination of unstained agar cultures is important. If the agar is transparent and thin, examination with oil immersion can be used successfully. To get a preparation appropriate for study, a piece of agar is cut out of the culture and placed on a slide. A coverslip is then placed on the agar and the space between slide and coverslip is filled with melted paraffin. Many phases of the growth can be followed in such preparations; for example, the germination of the large bodies was studied with this method. Oerskov used this method extensively and gave directions for it (*16*). The unstained preparations are not sufficient in themselves, however. Some processes observed on agar plates can be reproduced only with difficulty in slide cultures, and cultures on opaque media cannot be studied. Most of the interesting cultures were discovered on plates inoculated with routine bacteriological specimens, and they would have been missed without a method applicable to opaque media. Progress in the study of large bodies is due in a large measure to the application of stains to the agar preparations. The agar culture is mounted in the same way as for unstained preparations, but the coverslip carries a stain. The simplest method is to evaporate an alcoholic solution of methylene blue and Azure II on the coverslip (*3*). The staining is stronger if, in addition to the methylene blue, a small amount of $Na_2CO_3$ or $Na_3PO_4$ is present on the coverslip. While the colonies remain small, all bacterial organisms, including the large bodies and L type of colonies, are stained bright blue. In these preparations impurities in the agar, and dead autolysed bacteria, stain with a pink hue. The method can be applied to quite opaque media, such as blood agar, if the agar is cut into a thin slice. The making of these preparations requires less time than a smear, and they show with minimal distortion every organism developing in the culture. The large bodies and the L type of colonies, which in smears are usually entirely distorted and cannot be recognized, are clearly visible in all their morphological detail. The development of a bacterial colony is as clearly visible in these preparations as the details of a fungus colony in unstained preparation. The bacteria require staining in order to be seen because they are much smaller than the fungi. Most of the observations concerning the derivation of the dif-

ferent forms from each other have been made possible by the availability of this rapid and accurate method, which permits the observation of agar cultures with oil immersion. The majority of the photographs shown in the accompanying figures were made from stained agar preparations.

### OBSERVATIONS ON PROTEUS

The study of the phenomena reviewed above is in an early stage, and much new information can be expected from a more intensive investigation of the species already studied. It appears of great importance, however, to extend the investigation to as many other species as possible. Advance depends in a large measure on finding strains appropriate for study. In different species, and even in different strains of the same species, different aspects of the above processes are more readily studied. Study of saprophytic strains appears especially promising. All strains thus far studied were isolated from pathological processes and, on artificial media, were not in their natural environments. A natural environment probably can be realized more easily with saprophytes. Proteus was selected for study with this consideration in mind and also because a few large bodies had been seen in cultures of many strains. The study of Proteus brought an unexpected result, which promises to be of importance in the further study of the problems connected with large bodies.

The characteristic property of Proteus is its tendency to spread on the surface of the usual bacterial media. The cultures do not spread during the whole period of growth but only in certain phases of it. After they are transplanted the bacilli develop in small forms like *B. coli* and produce at first a nonspreading colony. At a certain point in the development of a colony the bacilli at its edge grow into moderately long, thick filaments, which are abundantly provided with flagellae and are able to move on the surface of the medium. These filaments produce the spreading halo around the colony. After the spreading has proceeded for a time the filaments at the outer border of the halo stop moving, break up into short bacilli, and multiply in this form. The motile filaments appear again after a time, and periods of swarming and multiplication of short bacilli follow each other until the whole plate is covered. On soft agar the culture may spread over the whole plate without stopping. In broth, growth is in the form of small bacilli. According to this description, therefore, spreading on a dry agar surface depends on the appearance of bacterial forms markedly different in morphology and in many other properties from the usual bacillary form of the Proteus.

An occasional large body is visible in the spreading halo produced by most strains. Large bodies are more numerous and appear in clusters if mixtures of different strains are planted. The interaction between the strains can be most clearly observed at the line where the spreading halos originating from colonies of two different strains come in contact. If the strains are appropriate, the majority of the filaments in this zone are transformed into large bodies within a short time. The further development of these large bodies can be followed for a while at the site where they are produced, but can be studied more readily when transferred to fresh media. The majority of them develop within one or two hours into small bacterial colonies. This happens in the manner previously described in the case of Bacteroides. First the large body increases in size and becomes irregular in contour. Later it assumes a bizarre form and then breaks into irregular, often branching, fragments. After a few divisions these resume the usual bacillary form. A few large bodies develop differently. These produce tiny L-type colonies, which soon stop growing and disappear in 10 to 12 hours. The majority of large bodies in the transitional zone develop similarly, and often only a few remain visible after a few hours. Thus Proteus, at the meeting place of two appropriate strains, goes through a complex reproductive process formerly observed in a few single strains of other Gram-negative species. In these species transformation of the whole culture into large bodies is usually a transient property, and when this property disappears it cannot be restored. In Proteus this reproductive cycle occurs regularly when cultures of two appropriate strains meet.

Certain morphological details of the transformation process observed in Proteus should be noted. The large bodies are produced only from the long, motile filaments. They are not produced from the small bacillary forms before the spreading starts. The large bodies are produced from the filaments in a manner different from that observed in other bacteria. They are produced by a process which has been described under the name "plasmoptysis." A small droplet appears on the side of the filament, and may increase within a few minutes to the size of a fully developed large body. The filament at the same time becomes thin and flexible. Evidently the content of the filament flows out and produces the large body. The large body is not a drop of liquid, however. Its appearance and staining properties are similar to those of the large bodies in other species. It is firmly attached to the filament and it is a resistant structure. After the development of such large bodies the filaments continue to move. They carry the large bodies with them and even pull them through narrow passages between other bacteria, which collide with them, often with great force. The large bodies may be deformed, but regain their shape immediately; large bodies are seldom disrupted by mechanical injury. When disrupted they disappear without leaving a trace, apparently because they have no membrane like the bacteria. Occasionally, large bodies are produced in

the manner observed in other bacteria, by swelling of the bacterial filaments. This process was seen particularly during observation of transplants made from the contact zone of spreading colonies. It is not known at present whether the filaments of both strains are transformed into large bodies. Probably this is the case, because nearly all filaments in the zone of contact between the cultures develop into large bodies. At times filaments belonging to different strains can be identified by differences in length, and in these cases both seem to be involved. However, this point needs further study.

The subsequent development of these large bodies into bacterial colonies or tiny L-type colonies occurs in a way very similar to that observed in Bacteroides, and needs no further discussion here.

The production of large bodies at the contact zone of two cultures depends on the strains and on the stage of the growth. Large bodies are produced only if spreading filaments meet spreading filaments. Even if the strains are appropriate, the spread of filaments of one strain into the bacillary forms of the other produces no large bodies. Inoculation of the filaments of one into broth cultures of the other is also without result. The meeting of different colonies of the same strain does not result in the formation of large bodies. Some strains are apt to produce large bodies if they meet the cultures of practically any other strain; some only if they meet cultures of a few special strains. The thirty strains that were studied most completely could not be divided into groups of similar strains which would not react among themselves and which would react with members of other groups.

Antagonism between different strains of Proteus is apparent on agar plates. Cultures of most strains will not grow into each other, and a noticeable line of demarkation remains between them even after incubation for several days. On the other hand, starting from colonies of the same strain, spreading covers the whole medium uniformly. As was mentioned previously, this demarkation line between different strains in fresh cultures contains many large bodies. Later most of these disappear, developing into bacteria. The cultures usually exert no influence on each other before they actually meet, but sometimes the spreading halos stop before meeting and a narrow, uncovered zone of agar remains between them. In other directions they continue to grow. This observation indicates that antagonism is caused at least partially by dissolved and diffusible substances. These substances do not spread far in the medium. If a piece of agar on which Proteus has grown is cut out and inverted, another strain will grow in spreading colonies on the reverse side. Large bodies are produced only by actual contact between the bacteria and not by the antagonistic action between two cultures. We have no information about whether the influences on which antagonism and production of large bodies depend

are the same. The filaments of both cultures when they come in contact remain actively motile even after the large bodies are formed. This, together with the viability of the large bodies, indicates that the filaments are not injured seriously. The different phases in the development of Proteus organisms, such as the appearance of spreading filaments, the arrest of their motility, their segmentation or the production of large bodies, occur at the same time in a large number of bacteria. This suggests that these processes are induced by common influences affecting the bacteria, probably by the appearance of certain substances in their surroundings.

That transformation of the filaments into large bodies is caused by influences of this kind is indicated also by other observations of the type already mentioned in the discussion of other species of bacteria. In some strains, transfer of the spreading filaments to tap water induces the transformation of some, or even of the majority, into large bodies. These are produced in the same way as in mixed cultures, by "plasmoptysis," and have a similar appearance. After some time most of them are transformed into ghost cells and become almost invisible. The small bacillary forms remain motile and multiply when transferred to tap water. The majority of the large bodies formed in tap water, even if transplanted immediately, are not viable. A few develop into tiny L-type colonies, and occasional ones develop into bacterial colonies. Surprisingly, most of the bacterial filaments that are not transformed also lose their viability. Of the numerous toxic salts that produce large bodies in bacteria, $HgCl_2$ was studied. A low concentration of this substance in broth (0.0025%) exerts no visible influence on the small forms of Proteus; they remain motile and continue to multiply. The spreading filaments are profoundly affected by a similar concentration. They stop moving, and the majority of them are transformed into large bodies. The bodies so produced are smaller than those developing in mixed cultures, and, even if they are immediately transplanted, fail to show any further development. If small bacillary forms are present, they grow readily in the transplants. The experiment with $HgCl_2$ is incomplete because detoxification with $H_2S$ was not tried. Phenol does not induce the transformation of the filaments into large bodies.

The large, spreading filaments of the Proteus are more sensitive to injury under various circumstances than the small bacterial forms, and they often react by transformation into large bodies. This reaction is one of the natural functions of the filaments, and the supposition that toxic influences start a natural process in an imperfect form applies very well to the Proteus. It is of interest to note that the ability to develop into L type of colonies is more persistent than the ability to develop into bacteria.

The most important observation made with Proteus was of the development of viable large bodies in the zone of contact of two strains. They develop from bacteria which are morphologically different from the usual bacilli and which are adapted to a special function, swarming. The filaments react to injuries of various kinds in the same way as to contact with another strain, but the large bodies so produced either are not viable or else have a very restricted viability. Initiation of a special type of reproductive process by interaction of two strains suggests sexuality. The reproduction of large bodies at the contact of two strains presents at least a superficial similarity to the conjugation and production of sexual spores in the zone of contact of fungus cultures. The sexual nature of the large bodies is suggested also by other observations. It was indicated previously that the L type of growth in bacteria presents surprising similarities to sexual forms of yeasts. A further observation suggesting a sexual process was that in one Bacteroides strain the large bodies developed not from one but from two neighboring individual bacteria. As has been previously mentioned, no sexual process of the type occurring in higher organisms—i.e., the union of cells—was observed during the formation of large bodies in Proteus or during their subsequent development. This does not mean that consideration of the sexual nature of these processes should be abandoned. The essence of sexuality is the exchange of hereditary properties between different strains, and there are observations which indicate that such a process occurs in bacteria without union of cells. A very complex hereditary characteristic, the production of type-specific capsules involving most important pathological and immunological characteristics of the pneumococcus, can be transferred from one type to another. According to the observation of McCarty and Avery (*14, 15*), a substance without apparent organization, probably a small fraction of the desoxyribonucleic acid liberated by the autolysis of the cocci, is responsible for the process. If similar processes occur under normal conditions of bacterial life (for example, autolysis is a constant accompaniment of the transformation into large bodies), then bacteria possess a mechanism for the exchange of hereditary characteristics, but it is different from the usual sexual processes. These considerations suggest the comparison of the characteristic properties of the strains that produce the large bodies with those of the strains originating from these bodies. Observations on this point may give valuable information concerning the significance of the complex reproductive processes observed in Proteus and in other Gram-negative bacilli.

## SUMMARY

A complex reproductive process, consisting of the swelling of bacteria into large, round bodies and the reproduction of bacteria either directly in them or by the intermediary of a soft granular growth ($L_1$), was observed in *Streptobacillus moniliformis*. Similar processes in a less complete form were observed in many other Gram-negative bacteria. In most species only a few exceptional strains exhibited these processes during a short period after isolation. In Proteus this complex reproductive process appears regularly when spreading cultures of appropriate strains come in contact. No union of cells is involved in this process. The fact that in Proteus two strains are involved offers an opportunity to study the problem of whether admixture and differentiation of hereditary characteristics occurs during this complex reproductive process and whether or not it is essentially a sexual process.

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# VARIATIONS IN ANTIGENIC PROPERTIES
## OF BACTERIA

RENÉ J. DUBOS

Bacteria are complex organisms, made up of a multiplicity of structures and chemical constituents. When introduced into animals, many of these (but not all) elicit the production of specific antibodies—modified serum globulins, which are capable of reacting with the injected material. In consequence, the injection of one type of bacterial cell usually results in the production of several species of antibodies—each of which combines selectively with one particular cellular constituent. Determination of the antigenic structure of a certain bacterial type consists in the recognition, isolation, and identification of the cellular constituents—the antigens—which are detected by these immunochemical reactions.

It will be apparent that the use of immunochemical procedures can contribute much to the study of the structure of bacterial cells. In principle, the method involves the preparation of antibodies specific for each one of the chemical constituents of the cell, the use of these antibodies as specific reagents for the detection and preparation in pure form of the corresponding cellular constituents, and finally the interpretation of antigen-antibody reactions in an attempt to define the relative positions occupied by these components in the living cell. In other words, antibodies can be considered as tools—as micro-reagents—for the detection and localization of certain cellular objects—the antigens—many of which would remain undetected by the classical cytological methods.

The subject assigned to me for discussion, "Variation in antigenic properties of bacteria," could therefore read "Variations in those cellular constituents that can be detected by immunochemical methods." Unfortunately, these methods are often cumbersome and unreliable, and should be used by the geneticist for the study of cellular variations only where other simpler and more direct procedures are not available. In the present discussion, I shall often substitute the words .capsule, flagella, cell wall, etc., for the expressions capsular antigen, flagellar antigen, surface antigen, etc., in order to impress upon you that antigens are cellular constituents, and that it is at times more convenient to observe them by classical cytological techniques than to detect their presence by immunochemical reactions.

It has been known since the beginnings of the science of bacteriology that the morphological appearance and the chemical composition of any given bacterial species are not constant, permanent properties. In the morphological domain, Pasteur (36) standardized techniques for the production of asporogenous forms of the anthrax bacillus. Colonial variation in vibrios was recognized as early as 1888 (19), and beautiful photographs illustrating variations in cellular and colonial morphology were published by Beijerinck long before rough and smooth colony variants received widespread recognition (7). That those morphological modifications are reflected in the type of antibodies elicited by the different variant bacterial forms has been recognized since 1903, when it was discovered that motile cultures of the hog-cholera bacillus give rise to nonmotile variants (42). It was found possible to analyze the immunological behavior of this motile organism in terms of two independent antigenic constituents, one present in the flagella, the other in the soma of the cell. The corresponding antiserum contains two different antibodies, only one of which reacts with the nonmotile bacillus, this variant having lost its flagellar antigen. As might be expected, an antiserum prepared against the nonmotile strain can react with both variants but the agglutination produced is of the slowly developing granular type due only to the antibody toward the somatic constituent.

The phenomenon of antigenic variation, and its relation to morphological variations, have now been recognized in all species of bacteria and will be illustrated again during this meeting by the case of the capsular variation in the pneumococcus group. It would not be possible or profitable, therefore, to present here a historical review of the subject. Instead, we shall select a few examples to illustrate the different modalities of the phenomenon and to state some of the hypotheses that have been formulated to account for it.

At the outset, however, it should be emphasized that the data concerning bacterial variation were obtained by investigators interested in the descriptive characters of bacterial cultures and in immunological and immunochemical reactions. They provide little or no information concerning quantitative variations in cell populations or the genetical factors involved in these variations. The bacteriologist, therefore, can only describe qualitative manifestations of variations, and it will be necessary for the geneticist to devise new experiments according to his own criteria if he wishes to utilize bacteriological material to analyze problems of heredity.

As already mentioned, antigenic variation can affect a great variety of cellular constituents. It is

likely that one could demonstrate by immunochemical analysis that certain functional groups of the cells—enzymes for example—can function as antigens and undergo variation as other antigens do. In fact, it is known that the α toxin of clostridia is a lecithinase and can stimulate the production of a specific antitoxin which neutralizes its enzymatic action; moreover, the amount of lecithinase (α toxin) produced by one given strain of Clostridium varies enormously under different experimental conditions. Granted, therefore, that antigenic variation of functional, metabolic cellular constituents may become a fruitful field of investigation in the future, we shall nevertheless limit our discussion to examples of variations affecting structural constituents of the cell.

Since there does not yet exist any convincing basis for a rational classification of the known examples of antigenic variation, we shall select cases illustrating three experimental manifestations of the phenomenon: (a) antigenic variations readily induced by physicochemical changes in the environment and readily reversible upon returning the environment to its original state; (b) modification of the antigenic specificity of a given cellular structure in the course of growth, a phenomenon known as "phase variation"; (c) loss or acquisition of a given cellular structure, reflected in loss or acquisition of the corresponding antigenic characteristic—this variation, often of a more or less permanent nature, has been described under the name of "bacterial dissociation."

Let us state again that this division of the material is only for convenience of presentation and does not imply any suggestion as to the fundamental nature of the reactions involved.

### ANTIGENIC VARIATIONS INDUCED BY PHYSICO-CHEMICAL CHANGES IN THE ENVIRONMENT

It has long been known that capsule formation is stimulated when the anthrax bacillus is grown in animal tissues, and when organisms of the colon-typhoid group are cultivated at low temperatures (10° to 20° C.) in the presence of fermentable carbohydrates (*37, 34*). In the case of streptococci of group H and of certain bacilli, the addition to the medium of large concentrations of sucrose or raffinose results in the production by the bacteria of capsular polysaccharides with well-defined immunological properties, which do not appear in the absence of these sugars. This phenomenon illustrates in a most striking manner the relation between composition of the culture medium and production of serologically specific bacterial substances (*35, 21*). So little is known of the influence of the environment on capsule production that it cannot be analyzed at the present time. In some cases, the medium probably favors the selection of a normally occurring capsule-producing mutant. In other cases, the presence of certain nutrients may be required

as building stones for the production of the capsular substance. In any event, one of the most important characteristics of this type of antigenic variation is its immediate reversibility, the cell returnng to its original state as soon as removed from the environment that induced the variation.

### PHASE VARIATION

We have already mentioned that motile, flagellated strains of bacteria can give rise to nonmotile, nonflagellated variants, and that this loss is often of a permanent character similar to a mutation. There is another type of variation, which results not in the loss of the flagella but in a reversible change in their chemical composition reflected in a reversible change in their antigenic specificity. This phenomenon has been described under the name of "phase variation of the flagellar antigens" (*1*).

Thus, when a subculture from a young colony of a biphasic salmonella (i.e., one capable of producing two different flagellar antigens) is incubated, all the flagella in the culture are antigenically identical for a few hours, but thereafter both types of flagellar antigen are found in the same culture. The terms "specific" and "nonspecific" flagellar phases were introduced to describe this situation; but in reality the antigens of either phase occur in various types, although it is true that the "specific" antigens are generally restricted to a smaller number of types. If repeated plate cultures are prepared from a strain in the specific flagella phase, bacilli in the nonspecific, group phase appear sooner or later in the culture. Similarly, a strain in the group phase gives rise to the specific phase by continuous cultivation (*1*).

By growth in homologous antiserum, on the other hand, it is often possible to reveal the existence of a second, hitherto unsuspected flagellar specificity in certain strains known under natural conditions only in the monophasic form—i.e., with only one flagellar antigen. This has been achieved, for instance, with *E. typhosa*, and the artificial phase culture can be reverted to the typical monophasic form. Thus, the variation from one flagellar phase to another can occur in either direction, and the ready reversibility of the reaction contributes greatly to its interest, especially since it can proceed in cultures isolated from single cells (*41*).

In other words, organisms that appeared to be permanently in the specific phase could be induced to change to the group phase, which then also seemed to be permanent until the organisms were again transformed into the specific phase by growing them in group serum (*8, 12, 13, 20, 30, 40, 45*). The experimental transformation of *S. salinatis* into a culture that cannot be differentiated from *S. sandiego* illustrates the interest of the method.

When *S. salinatis*, containing flagellar antigens d, e, h-d, e, n, $Z_{15}$, is grown in the presence of a serum

containing an antibody active against the d component, one obtains a new culture deficient in this component and possessing the flagellar formula e, h-e, n, $Z_{15}$. As already stated, the new culture thus artificially produced is identical in all respects with *S. sandiego,* a strain isolated from a natural source. This laboratory transformation of one naturally occurring type into another gives insight into the many changes in antigenic structure that are probably constantly taking place in nature. It suggests that the immunological complexity of the salmonella is due to the fact that the members of this bacterial group are in an unstable state and are undergoing evolution at a rapid rate *(17, 46, 47)*.

The phenomenon of phase variation—i.e., the change in immunochemical specificity of the flagellar antigens of one given species—can therefore be observed under two different sets of conditions. On the one hand, it occurs spontaneously in the course of growth of the culture—a new antigenic characteristic, which was not present in the early stages of growth, appearing in the later stages. On the other hand, it can be induced and directed in a predictable manner by growing an organism in the presence of an antibody specific for the flagellar antigen possessed by this organism. This procedure inhibits the production of the antigen in the newly formed cells and elicits in some cases the appearance of another flagellar constituent which the culture was potentially capable of producing.

## BACTERIAL DISSOCIATION

Practically all, if not all, bacterial species have been observed to lose certain of their characteristic morphological structures and antigenic properties without their viability being affected thereby. These loss variations occur usually under unpredictable and uncontrollable conditions.

Thus, we have already mentioned that sporulated cultures of the anthrax bacillus can become asporogenous and that motile flagellated species can lose their flagella and flagellar antigens and become nonmotile. Although the Smooth → Rough variation was first clearly described in Gram-negative bacilli by changes in colonial and cellular morphology, by increased spontaneous agglutination of the cell suspension, and by decrease of virulence, it is now more commonly defined in terms of the loss of the O polysaccharide antigen *(2, 3, 4)*. The O antigen is specific for each bacterial type; it appears to occupy a superficial position in the cell, masking a variety of other antigenic components which become exposed only after removal of this polysaccharide by mechanical or chemical means, or when the culture undergoes variation (S → R). Other surface antigens (Vi) exist in certain virulent cultures of Gram-negative bacilli, and can also be lost independently of the O antigen *(18)*. Similarly, encapsulated pneumococci can lose

the ability to produce the serologically specific capsular substance *(6)*; streptococci growing in the form of "matt" colonies characterized by the production of the type-specific M protein can change to the nonspecific "glossy" phase, which does not contain the M protein *(31)*.

We have mentioned only a few of the many examples of loss of type-specific antigenicity that have been observed in bacterial species. Enough has been said, however, to illustrate the fact that the pattern of variation, and the terminology employed to describe it, vary so greatly from one case to another that we are still very far from being able to formulate a unified concept of the phenomenon. Moreover, the examples that have been cited deal especially with the type-specific bacterial antigens, cellular components which have been particularly well studied because they are of so much practical importance in the analysis of virulence and infection. Variation in antigenic structure, however, can go much further than is revealed by the classical changes typified by the Mucoid → Nonmucoid, Smooth → Rough, Matt → Glossy, etc., types of variation.

Thus, in the case of pneumococci and streptococci, there have been recognized in addition to the nonspecific (rough) colonies other forms which appear further degraded and which consist of cells no longer coccus-shaped but exhibiting instead a bacillary or even mycelial morphology.

Old strains of salmonella kept for many years on artificial media are often found to possess neither the smooth O nor the rough antigen. They seem to correspond to a further step in the way of loss variation, and have been called the ρ variants. From these ρ forms, as well as from the R and S forms, one can obtain by extraction with 95% to 97% alcohol acidified with HCl, a protein that has been designated as Q. The Q protein is antigenic and gives rise to sera which agglutinate the ρ forms well and the R forms slowly, but not the S forms— although Q is also present in the latter *(48)*. After removing from the cell the O, R and Q antigens, one can still extract by 75% acid alcohol another antigenic protein (T), which also gives rise to antisera capable of agglutinating the R and ρ but not the S forms *(49)*.

Although the phenomena of bacterial dissociation thus embrace a large variety of variations—either irreversible or only reversible with difficulty—of many different types of cellular components, little can be said concerning their significance for the biology of the bacterial cell. Some workers believe that, in spite of their apparent diversity, they can be described in terms of a unified terminology, and correspond to normal stages in the developmental life cycle of the individual organisms *(23, 24, 25)*.

"Any bacterial culture, arising from a single cell and developing on common culture mediums, may manifest in the course of time many diverse cell

forms and culture growths. Those most frequently observed are the mucoid, smooth and rough, while a gonidial phase (G phase) and a diphtheroid (D phase) are present in several species" (26). Furthermore: "What might be considered a sort of ontogeny for the individual of any bacterial species would comprise progressive development through the culture phases G, S and R. Morphologically, they begin as the tiniest of coccus forms (the G) and progress to the coccus, chained, and filamentous structures; the latter being characteristic of the rough phase culture in all families above the Coccaceae. An interesting parallel exists with reference to the great so-called family groups of bacteria. The progression of the micrococci to the Coccaceae, to the Bacillaceae, to the Bacteriaceae—the Mycobacteriaceae—the Actinomycetaceae—etc., exemplifies it" (26).

Thus Haeckel's aphorism that ontogeny recapitulates phylogeny would apply to bacteria, and the different forms in which we know bacteria— smooth, rough, etc.—would be only more or less permanently stabilized ontogenic phases of polyphasic organisms (33).

Other workers, on the contrary, are inclined to consider that the changes in antigenic structure described under the heading of bacterial dissociation constitute only examples of discontinuous variations of independent structural characters. In fact, recent studies of dissociation in *Brucella abortus* have led to the conclusion that the phenomenon can be "interpreted in terms of the spontaneous appearance of variants (mutants) and their subsequent establishment under the control of the inherent and environmental factors which govern population dynamics" (9). Analysis of the mechanism of variation is rendered more difficult by the paucity of quantitative data concerning the frequency of occurrence of the phenomenon, a situation due in part to the impossibility of carrying out statistical studies within a bacterial population in which the different individuals cannot be readily differentiated. Except in a very few suggestive cases, such as the production of mucoid material by paratyphoid B bacilli (27), and the production of pigment by *Serratia marcescens* (32), there is no evidence that bacterial variation behaves according to Mendelian laws.

Quantitative studies of variation are somewhat more satisfactory in the case of bacterial cultures sufficiently unstable to give rise to a large and predictable number of variant forms.

Thus, certain strains of *Salmonella aertrycke* have the property of constantly giving rise to two different colonial forms in detectable and predictable proportions (15). One of the unstable bacterial cultures studied appears as a mixture of two types of cells: those breeding true—i.e., yielding a pure stable culture—and those constantly giving rise to the mixed culture. The terms "pure" and "stable" are employed in this connection in a relative rather than absolute sense. They imply that if the "stable" cultures exhibit variation, it is so rare as not to be detectable under ordinary cultural conditions. As long as environmental factors remain unchanged, the ratio of the variant to the parent type is found to remain constant despite repeated selection of the latter, this ratio being, for instance, 41% with a certain form, 19% with another, and less than 2% with a third. Altered environment, in certain instances, produces a temporary change in the characteristic ratio for each form, but on reculturing in the original environment, the ratio always reverts to its original value. The 2% ratio, characteristic of one of the forms, is very close to the lower limit at which it is possible to recognize an unstable organism with the method used. It is apparent, therefore, that a so-called pure or stable organism could really be unstable and capable of undergoing variation, but that the percentage of variants given off could be so small as to escape detection during ordinary cultivation (15).

These facts suggest that, in certain cases at least, bacterial variation is based on a fixed mechanism and that each organism has an inherent potentiality for producing a variant form once in a given number of divisions.

Just as phase variation of flagellar antigens can be induced and specifically directed by growth of the organism in specific antiflagellar sera, the changes in antigenic structure corresponding to bacterial dissociation can be brought about by growth in the corresponding immune antiserum.

It has been repeatedly shown that pneumococci growing in media containing the homologous, specific, anticapsular antibody change more or less rapidly to the nonspecific, noncapsulated growth phase (22, 38, 39, 44). The nonspecific, noncapsulated form of Friedländer's bacillus can readily be produced by a similar technique (28, 29), and the method also applies to the conversion of the smooth O forms of the salmonella into the rough nonspecific variants (5). The converse change from the nonspecific to the specific form has also been obtained by growth in serum directed against the nonspecific phase; pneumococci are thus changed from the noncapsulated to the mucoid state (14). Interchange in both directions has also been obtained by growing in their appropriate antisera two variants of *B. subtilis*, one motile and forming typical smooth colonies, the other nonmotile and growing in long chains forming medusa-head colonies (43).

The changes induced by antisera exhibit great selectivity, determined by their immunological specificity. Thus, a serum directed against the S form of *S. enteritidis* (Gaertner), an organism which possesses antigen IX in common with *E. typhosa*, is also capable of causing the latter organism to change from the S to the R form. When, however, the serum is heated at 70° C., or absorbed

with the former culture in order to destroy or remove the specific antibody, it loses its ability to induce transformation of either organism (40).

Any interpretation of the changes in antigenic structure induced by appropriate antisera must take into account the fact that, in many cases, the same changes can occur under other unspecific influences or even spontaneously without any detectable influence of the environment. When, for example, smooth strains of typhoid or paratyphoid A bacilli are subcultured in different media, it is found that R forms are produced most rapidly (ten days) in media containing immune sera directed against the S forms. R forms also appear, however, in other media devoid of specific antibody, although the change in these cases requires many weeks or even months (40). As already stated, phase variation of the flagellar antigens is also a normal occurrence (1). Although further analysis of these phenomena is therefore required to establish the mechanism of action of the antibodies, it appears possible, and indeed likely, that these reagents only accelerate a process of change which goes on continuously in any culture, and that their action may consist only in a selective inhibition of the forms with which they react immunologically.

## CONCLUSION

Great progress has been made towards correlating the variations in antigenic properties of bacteria with certain specific changes in the cellular architecture, but no accurate information is available to elucidate the mechanism of the phenomenon. How frequent is the occurrence of variation in the life of a clone? Do the changes take place gradually in each individual cell, or do they reach their final expression from one cellular division to the next? How reversible are the variations, and what are the factors that affect reversibility? What are the metabolic reactions that determine changes in antigenic structure? These, and many others, are questions to which there is no answer at the present time.

Moreover, all attempts to formulate a biological theory of the mechanism of antigenic variations are paralyzed by the suspicion that the process of cellular division in bacteria is not limited to simple binary fission but may at times take place through a more complex developmental cycle (16). In the absence of some process of fusion and segregation, it is difficult to account at the present time for the observed phenomena of bacterial variation; whereas, granting the assumption of the occurrence in bacteria of genetic impurity with some mechanism of segregation, the data of variability might fall in line with classical genetical conceptions. True evidence of fusion would permit the interpretation of variation in terms of combination and segregation. Production of variants would then be the expression of segregation from genetically complex parentage,

while there would remain the additional possibility of the occurrence, at unpredictable intervals, of true mutations not depending upon segregation and recombination (10, 11).

Thus, the field of antigenic variation appears at first inspection to present many obstacles for genetical analysis. It deals with changes which are difficult to observe, even more difficult to measure, and the biological and chemical mechanism of which are completely unknown. And yet it illustrates in a vivid manner one of the most interesting phenomena revealed by the study of microorganisms. The bacterial cell can successfully continue to exist and multiply as an independent living object after having lost a great variety of structures and functions which had appeared to constitute important components and attributes of the "normal" parent form. These structures and functions can be lost and regained independently of each other. It is even possible to substitute experimentally one character for another—to cause, for instance, a strain of pneumococcus to produce, and to transfer to its progeny the ability to produce, a polysaccharide different from the one it had been known to synthesize before. Not only does the cell appear as an integrated complex of independent characters, but it is possible to substitute for one of these characters another one homologous, but different, without interfering essentially with cellular organization.

With this discovery, to be discussed today by Dr. McCarty, bacterial variation passes from the collector's box of the naturalist to the sophisticated atmosphere of the biochemical laboratory. One may wonder whether the geneticist will not arrive too late to introduce his jargon into bacteriology.

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## DISCUSSION

MELLON: May I elaborate somewhat on Dr. Dubos's comment to the effect that Dr. Dienes has been the champion in this country of the so-called "large bodies" produced by bacteria. To my personal knowledge, the essentials of the Dienes phenomenon—*viz.*, the freeing of intracellular units from these bodies, which are then capable of multiplication—was observed by ourselves in 1917 and subsequently. Some of these observations were published shortly afterwards. I shall only refer to one

of several of our studies dealing with the manifold potentialities of these structures.

In this instance the "large bodies" appeared first in a blood-culture flask, occurring as small clumps on top of the sedimented red cells. They were removed with a fine capillary pipette, but without disturbing the clear supernatant broth. When stained, the intracellular coccoidal structures could be seen. This fact, combined with the observation that within the following hour the supernatant became densely turbid with a Staphylococcus-like organism, caused us to hypothecate a "frantic multiplication" as the result of liberation of the intracellular coccoids.

This idea was supported by direct warm-stage observations. Thus, over a 24-hour period these "large bodies" (or giant cocci as we then called them) showed initial signs of activity by developing 4 or 5 oval, refringent structures. Some time later their thick outlines became indistinct and in their place gradually appeared minute forms whose motility was so great that the whole cell, now swollen to nearly twice its diameter, oscillated perceptibly. Shortly thereafter it ruptured, releasing the highly motile forms, singly or in packet-shaped groups, into the surrounding medum.

The minute size of these liberated forms suggested their filterability; confirmation was readily obtained by passing the patient's blood serum through a Berkefeld N bougie, as the result of which the Staphylococcus-like organism grew luxuriantly in the filtrate after 24 hours. That no technical error was involved was evidenced by the fact that repeated attempts to pass this coccus through the identical bougie failed completely. Nevertheless, this bougie was still permeable to the filterable form present in the blood. The cocci grown in the filtrate had the remarkable quality of giving rise to the large bodies in profusion, and they were the ones subjected to warm-stage study.

In this connection it should be recalled that Dienes's "large bodies"—particularly the ones he described for the pleuro-pneumonia visible-growth phase—are also capable of yielding a filterable phase, which is the well-known L form of this organism. Indeed this phase was one of the earliest of the viruses described, although it may not receive allocation with the true viruses now. In other words, Diennes's large bodies have this as a second mode of germination in addition to the more conventional one referred to by Dr. Dubos (*viz.*, that of regenerating the parent phase of the organism).

Nor do these phenomena exhaust the potentialities of these interesting structures. At about this same time we showed that in a diptheroid bacillus from Hodgkin's Disease, typically formed coccoidal spores stemming from its branching phase, underwent a gradual tetrad segmentation that resulted in their germination as tetrads and diplo-streptococci. Moreover, such coccoidal structures are capable of germinating as such, thus yielding a new culture phase. This has been observed more frequently in those of intermediate or smaller size. There is much more that I could say of the potentialities of these forms, if time permitted.

Before stopping, however, I wish to ask Dr. Dubos whether the Type M protein which Mrs. Lancefield has isolated from the matt phase of streptococci, is also in the mucoid phase, which we think of as lying next door to the matt?

Dubos: Dr. Mellon's contributions to the problem of an atypical mode of reproduction of the bacterial cell are well known to bacteriologists. Several other workers have reported similar observations, and it is only lack of time that prevented me from giving a more extended discussion of this topic. On the other hand, it is unfortunate that neither Dr. Mellon nor any other investigator in the field has been able in the past to describe methods which would permit the production and demonstration of the "large bodies" or other atypical reproduction forms and cycles in a predictable manner. As Dr. Mellon himself has forcefully emphasized, the phenomena had been observed only for a limited period of time with a few strains recently isolated from pathological material. These strains were found to lose rapidly the ability to produce atypical forms, whatever the method of preservation or cultivation of the culture. I have singled out Dr. Dienes's recent observation of the production of reproductive "large bodies" because, for the first time, we appear to be dealing with a reproducible, predictable phenomenon, susceptible to experimental analysis.

With reference to Dr. Mellon's last question, I need only state that the factors responsible for the "glossy," matt, and mucoid characters of hemolytic streptococci of group A appear to vary independently of one another and can occur in any possible combination in any given strain.

# SPONTANEOUS MUTATIONS
## IN BACTERIAL VIRUSES

A. D. HERSHEY

It is reasonable to suppose that among the viruses we observe the operation of the principles of heredity at an elementary level of organization not far removed from that of the gene itself (3). Whether this supposition proves correct or incorrect, it provides sufficient justification for a thorough inquiry into the genetic behavior of the viruses.

There has long been reason to believe that heritable variations occur in all kinds of virus; the multiplicity of closely related strains found in nature (11) leads immediately to this conclusion. Once this inference has been drawn, observations on the variation phenomenon itself can contribute little fundamentally new information until the purely technical difficulties of handling the materials can be overcome. The minimal requirements are:

First, the propagation of clones from single viral particles.

Second, evidence that the particles themselves are not mixed aggregates.

Third, methods of recognizing and selecting mutants arising in the clones; for many purposes, these methods must be quantitative.

At the present time these requirements, especially the first and the third, are not easily met for any of the plant or animal viruses (2, 11, 24). For bacterial viruses, the first requirement is fulfilled, since the plaque count is proportional to viral concentration, even when the absolute infectivity is low (15), and since in favorable cases the infectivity itself approaches unity. This last can be seen both from the correlation between weight of purified virus and infectivity (17), and that between visible particles and infectivity (22). The second requirement is satisfactorily established by application of the electron microscope (22), and also from the homogeneity of size determined by physical methods (16). With the methods of viral genetics proper, I shall have to deal at length in what follows. For the reasons already stated, this will have to do mainly with experiments using bacterial viruses.

Before asking questions about the mechanics of viral heredity, one must know how to recognize unit characters, and how to analyze their mutual relations. It is my principal assignment here to tell how we are learning to do this. I shall begin with some general remarks concerning methodology, and some requisite definitions.

Owing to the nature of the material, our principal tools for the study of viral genetics are the analysis of mutational patterns and the measurement of mutation rates. For this reason alone, viral genetics is bound to differ in superficial appearance from classical genetics, where the principal tool is segregation. When we speak of independent genetic sites in the viral particle, we will mean loci that mutate independently of other loci. Their relation to the genetic locus recognized as a crossover unit is dubious to say the least, though this cannot be said to diminish their importance. But it must be kept in mind that genetic factors recognized to be independent by analysis of mutations in a virus, may prove to be structurally analogous to factors classified as alleles by segregation experiments with other materials.

Actually, this difference of method is probably not inherent in the materials. Analysis of mutational patterns in maize (29) reveals independent genetic factors associated with a single locus. In bacteria, the study of mutational patterns (10), and the transmission of genetic material exemplified by the transformation of pneumococcal types (1), may come to supplement each other. An analogy to the Griffith experiment, resulting in genetic modification of rabbit-fibroma virus (4), and seemingly related experiments with bacterial viruses to be described at this Symposium by Dr. Delbrück and myself, promise an equivalent of, or a substitute for, meiotic segregation in the genetic analysis of viruses.

### DEFINITIONS

To avoid possible confusion of ideas, I shall define a few words in the sense with which they will be used in this paper.

By *mutation,* I mean a change in any property of a virus which occurs in a single step from one recognizable type to another, both types breeding true in susceptible cells except for additional mutations. It should be noted that this definition includes all heritable changes known to occur among the viruses.

The term *genetic site* will refer to the hypothetical structural site of a mutation or set of alternative (allelic) mutations that occur independently of other known mutations. When the word locus is used in connection with viral genetics, it is necessarily the genetic site that is meant.

The term *allele* refers to any one of the alternative modifications permissible to a given genetic site, or to the corresponding phenotypic effect.

A *genetic complex* consists of the collective genetic sites, where there is reason to suspect more than one, responsible for a given hereditary character; *e.g.,* host specificity.

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Additional definitions have to do with the genetic notation (14) recently adopted by a group of workers all studying the same system of bacterial viruses acting on a certain strain of *Escherichia coli* known as B. *The viruses* are called T1, T2, etc., where T stands for type (10). *Viral mutants* are named according to a convention illustrated by "T1*h*," which is the generic symbol for mutants of T1 characterized by altered host specificity. With reference to this class of mutations, the symbol for wild-type T1 becomes T1*h*⁺. A given *h* mutant is further defined when necessary in terms of its host range, and the type of plaque it forms on the selective host. *Bacterial mutants*, derived from the sensitive strain B, and characterized by resistance to particular viruses, are named according to their resistance pattern; *e.g.*, B/1, 5, resistant to T1 and T5 (10). This symbol further implies that the mutant was selected by means of T1, and that its resistance resulted from a single mutational step. It should be stated here, too, that *the virus T2H*, to which I shall refer frequently, is a near relative of T2, whose mutational origin is unknown (14).

### Typical Mutations in Bacterial Viruses

Comparing the related bacterial viruses found in nature, one sees numerous points of difference, all of which must be genetically determined and subject to mutation. (This statement, which appears self-evident to us now, nevertheless expresses a comparatively novel point of view.) The more obvious of these are differences in host specificity, in type of plaque, and in antigenic specificity.

Heritable alterations of antigenic specificity have not been observed, either as an accompaniment to other mutations (14, 20) or among the survivors of the action of antiserum (unpublished experiments of the writer and of M. Delbrück). On the other hand, neither of these methods of selection has been adequately investigated. The question pertinent to genetics is whether antigenic alterations result from rare mutations independent of other mutations, or whether they result from the accumulation of successive mutations of any sort. This question cannot be answered from available information.

Mutations with respect to host specificity have been described several times (5, 13, 27). The first quantitative and systematic study was made by Luria (20, 21). He established that alterations in host specificity occur in a number of unrelated viruses, that they are the result of relatively frequent mutations occurring during the growth of the virus prior to its contact with the selective host, and that they probably occur intracellularly.

I have found further, in unpublished experiments partly in collaboration with Dr. Luria, that one can distinguish at least three host-range mutations of T1 and at least six of T2H, and that at least three can occur as the result of what seem to be single alternative mutational steps. The rate of one of

these in T2H has been measured with something approaching quantitative rigor; it occurs once in about $4 \times 10^8$ duplications of the virus (14). Some of the new mutations will be described in this paper.

A series of mutations affecting type of plaque has been reported by Sertic (28), and it now seems likely that his series included the mutation I want to speak of next, the so-called *r* mutation.

Before describing this mutation, it is necessary to refer to a peculiar phenomenon known as lysis-inhibition, first attentively noticed by Dr. Delbrück (personal communication) in connection with his one-step growth experiments. He found that when you infect all or most of the bacteria in a visibly turbid culture with virus T2, clearing does not occur for 5 or 6 hours, although a sample of this culture previously diluted in broth lyses promptly at 21 minutes, as shown by the rise in titer of virus. The delay is not due merely to crowding in the undiluted culture, for bacteria are promptly lysed by other phages under the same circumstances. It seems likely, from still uncompleted experiments of A. H. Doermann in Delbrück's laboratory, that lysis-inhibition is an effect of readsorption of viral progeny to the previously infected bacteria just as they are about to lyse. The more intimate mechanism of this effect remains unknown.

Lysis inhibition is observed only with T2 and its relatives; namely, T2, T4, and T6, and several others that I have tested. All these phages undergo mutation to forms that do not give lysis-inhibition. I have called these *r* mutants, the *r* being mnemonic for rapid lysis.

The *r* mutants can also be identified by their distinctive type of plaque (Fig. 4), and can be isolated readily from characteristic sectors of rapid lysis found with varying frequency in the zone of lysis-inhibition of wild-type plaques.

A rather thorough study of *r* mutation (14) has established the following facts.

(1) It occurs without change in host or antigenic specificity.

(2) The frequency of its occurrence in T2H is roughly one per thousand duplications of the virus. This frequency has since been found to vary in genetically different stocks.

(3) The reverse mutation occurs also, with a frequency of about $10^{-8}$ in T2H*r*. The recovered lysis-inhibitor is identical with the original in respect to host range, plaque type, and rate of mutation to *r*. Table 1 shows the frequency of *r* mutant found in clones of T2H*hr*⁺ and the corresponding back-mutant from T2H*hr*. The genetic factor *h* was carried in this experiment to exclude the possibility of contamination during the successive transfers required for the isolation of the back-mutant (14). The counts were made by sampling individual plaques from the respective viral stocks.

(4) During the propagation of mixtures of lysis-

inhibitor and $r$ mutant, the inhibitor replaces $r$. This accounts for the fact that the lysis-inhibiting viruses of this group are the wild type, in spite of the unfavorable balance of mutation rates. It also permits

TABLE 1. PROPORTION OF $r$ MUTANT IN CLONES OF T2H$h$

Back-mutants No. 1 and No. 2 are of independent origin. 1/2400 means one $r$ plaque found among 2400 examined from a single clone.

| (Wild-type clones) | (Clones of back-mutant No. 1) |
|---|---|
| 1/2400 | 1/2700 |
| 0/1600 | 0/3700 |
| 0/910 | 3/6100 |
| 1/1600 | 4/8300 |
| 2/1800 | 4/4900 |
| 0/800 | (Clones of back-mutant No. 2) |
| 2/1700 | 1/3500 |
| 2/4200 | 0/3300 |
| 0/2700 | 5/4400 |
|  | 0/3800 |
| Mean 1/2200 | 0/3000 |
|  | Mean 1/2400 |

the isolation of back-mutants, which would otherwise be impossible. Several kinds of experiment fail to explain how the selection of wild type during growth is brought about.

(5) The $r$ and $h$ mutations of T2H correspond to independent genetic sites. The evidence for this statement will appear in the following discussion of analytical methods.

## THE ANALYSIS OF GENETIC FACTORS

### Independent Phenotypes

When two mutations are found to be phenotypically independent—that is, when the expression of either one is not influenced by the occurrence of the other—the analysis is comparatively simple. This is true of mutations with respect to host range and lysis-inhibition in T2H. Fig. 1 shows the muta-
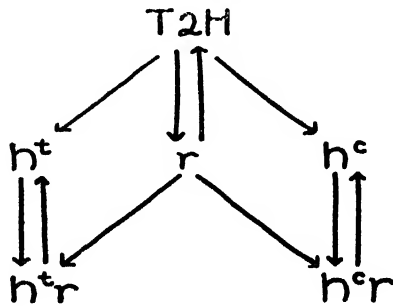


FIG. 1. Independent phenotypes in T2H.

tional pattern for this virus with respect to the phenotypes $r$ (rapid lysis), $h^t$ (turbid plaques on the bacterial strain B/2H resistant to T2H), and $h^o$ (clear plaques). It will be seen that mutation to $r$ and reversion to $r^+$ occur irrespective of the existing

$h$ phenotype, and without altering it. Similarly, the two $h$ mutations occur in either wild-type or $r$ stocks, without altering the character with respect to lysis-inhibition. The use of this method might be spoken of as *pattern analysis* of mutations.

The mutational pattern shown in Fig. 1 is sufficient evidence that host range is determined by one genetic site or genetic complex ($h$), and that lysis-inhibition is governed by a second genetic site or complex ($r$) independent of the first.

The inference just stated can also be tested quantitatively, by measuring the rates of parallel mutations as a function of the accompanying phenotype. This has been done for the mutation $h^+ \to h^c$ in $r$ and $r^+$ stocks of T2H. The rates turn out to be identical, confirming that the two kinds of mutation occur independently (*14*). This method I shall call *rate analysis*.

### Dependent Phenotypes

Alternative mutations yielding dependent phenotypes are also subject to rate analysis. Pattern analysis is not applicable, however, unless one of each pair of alternative mutations is demonstrably reversible. I shall discuss three cases.

The first is concerned with two alternative host-range mutations in T2H. By selecting from T2H virus active on the bacterial strain B/2H, one gets the mutant $h^c$ previously referred to, to which another bacterial strain B/2, 2H, $h^c$ (resistant to the original T2 as well as to T2H) is resistant. By selecting from T2H virus active on B/2, 2H, $h^c$ one gets another mutant $h^b$ active both on B/2H and B/2, 2H, $h^c$. One can also select from $h^c$ virus active on B/2, 2H, $h^c$, getting the mutant $h^c h^b$, which is not very different from $h^b$. All three mutants are distinguishable by quantitative test, using suitably chosen bacterial indicator strains (Table 2).

Suppose we want to determine whether $h^b$, $h^c$, and $h^c h^b$ (together with the wild type $h^+$) form alleles of a single genetic site, or whether $h^b$ and $h^c$

TABLE 2. DIFFERENTIATION OF HOST-RANGE MUTANTS OF T2H

The table shows the results of tests on seeded agar plates (*14*) using samples of virus taken with an inoculating needle directly from plaques. The samples are suspended in 1 ml. fluid, to contain about $10^6$ viral particles per drop. C means complete lysis, numbers refer to plaques, t means turbid plaques, and — indicates no effect.

| Phenotype | Bacterial strains | | |
|---|---|---|---|
|  | B/2H | B/2, 2H | B/2, 2H, $h^c$ |
| wild type | — | — | — |
| $h^t$ | C | — | — |
| $h^c$ | C | 500 | — |
| $h^b$ | 200 | 200 | 200 |
| $h^c h^b$ | C | C | 500 |
| $h^a$ | 500 | 500t | 100t |
| $h^c h^a$ | C | C | 50t |

are independent mutations combining to give $h^c h^b$. One obvious clue is the fact that the mutations $h^+ \rightarrow h^b$ and $h^o \rightarrow h^c h^b$ occur with similar frequency, which suggests that the genetic sites $h^o$ and $h^b$ are independent. Further analysis rests on the fact that back-mutations can be detected in the $h^b$ locus.

Both $h^b$ and $h^c h^b$ grow rather poorly on sensitive bacteria, perhaps because of poor adsorption. Whatever the reason for the poor growth, it is evident that if back-mutations (or other mutations leading to better adaptation to the bacterial host) occur, the new variants will quickly outgrow their progenitors. This is in fact what happens.

Starting with pure stocks of $h^c h^b$ (three stocks were tested, each originating from a different single plaque), 3 to 5 serial transfers in broth cultures of sensitive bacteria are sufficient to yield stocks in which the predominating type is phenotypically $h^c$. Some additional phenotypes, principally $h^c h^a$ listed in Table 2, also appear.

Similarly, serial transfer of initially pure stocks of $h^b$ quickly yields stocks containing phenotypic $h^+$. The mutant $h^b$ grows especially poorly on sensitive bacteria, forming turbid plaques in agar and causing little visible lysis in broth. Occasionally on the first inoculation from a single plaque into broth, and regularly on the second transfer, lysates of fair titer are obtained, but these always consist principally of the progeny of further mutants. Besides $h^+$, a variety of new phenotypes appear, most of them resembling the mutant $h^a$ listed in Table 2.

TABLE 3. BACK-MUTATIONS IN HOST-RANGE MUTANTS OF T2H

| No. of serial transfers | No. of isolates of phenotype | | | | | |
|---|---|---|---|---|---|---|
| | $h^b$ | $h^+$ | $h^a$ | $h^c h^b$ | $h^c$ | $h^c h^a$ |
| | (from $h^b$) | | | (from $h^c h^b$) | | |
| 1 | 10 | 0 | 0 | 10 | 0 | 0 |
| 2 | 4 | 3 | 3 | 6 | 3 | 1 |
| 3 | 2 | 5 | 3 | 5 | 4 | 1 |
| 4 | 0 | 6 | 4 | 1 | 9 | 0 |
| 5 | 3 | 5 | 2 | 1 | 9 | 0 |

These changes in composition of the population are easily followed by plating out samples of the successive stocks with sensitive bacteria, and then making host-range tests of the contents of a number of individual plaques in the manner shown in Table 2. These tests are entirely reliable, for if the contents of the single plaques are again plated out, and the tests are repeated with a number of these secondary plaques, the results always agree. Similarly, tests of a large number of plaques originating from the initial stocks of each type always agree.

The results of typical passage experiments are shown in Table 3, and the pattern of mutation brought out by means of them is diagramed in Fig. 2. It is found that the mutant $h^b$ readily goes back

to the phenotype $h^+$ from which it came, but never to $h^o$. Similarly, the mutant $h^c h^b$ readily goes back to the phenotype $h^o$ from which it came, but never to $h^+$. To the same effect, when new mutations occur, it is usually clear from the phenotype whether they have departed from $h^b$ or $h^c h^b$.
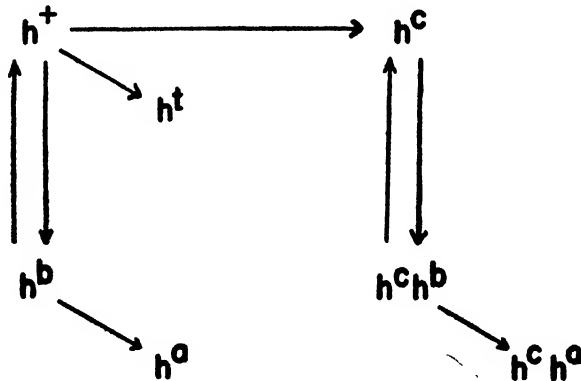


FIG. 2. Mutational pattern of T2H with respect to host range.

This behavior supports very well the conclusion, already suggested by rate analysis, that $h^b$ and $h^c$ are independent genetic factors, and that the parallel mutations could be more informatively represented by some such notation as $(hc)^+(hb)^+ \rightleftarrows (hc)^+(hb)$ and $(hc)(hb)^+ \rightleftarrows (hc)(hb)$.

TABLE 4. RATES OF MUTATION IN IDENTICAL PHENOTYPES OF DIFFERENT MUTATIONAL ORIGIN

Each count is the mean for five different stocks prepared from different single plaques of the specified viral type. The titers are means from a count of a pooled sample of the same five stocks. The mutant plaques are counted per drop (about 0.03 ml.) on the surface of seeded agar plates. A single count (100 plaques per drop on B/2H) has been omitted in calculating one of the averages.

| Phenotype | Origin | Titer$\times 10^9$ | Plaques on B/2H | Plaques on B/2, 2H, $h^o$ |
|---|---|---|---|---|
| $h^+$ | wild type | 20 | 7.4 | 2.2 |
| $h^+$ | $h^b$ | 11 | 7.2 | 1.2 |
| $h^c$ | wild type | 3.4 | — | 0.6 |
| $h^c$ | $h^c h^b$ | 9.3 | — | 1.2 |

It is of general interest, as well as important to the problem under discussion, to determine whether the phenotypically reversible mutations shown in Fig. 2 are authentic back-mutations; i.e., whether the identical phenotypes of different origin are genetically the same. In an attempt to answer this question I have again made use of rate analysis. Table 4 shows that the frequency of mutation to $h^b$, either from wild type or from back-mutant of $h^b$, is the same; that the frequency of mutation to $h^o$ is also the same for this pair; and that the frequency of mutation to $h^c h^b$ is the same whether one starts from $h^o$ derived from wild type, or derived

from $h^c h^b$. The table also shows, as previously stated, that the parallel mutations $h^+ \to h^b$ and $h^o \to h^c h^b$ occur at the same rate.

It is very likely therefore that mutation in the genetic site $h^b$ is truly reversible. Whether the mutation in $h^o$ is also reversible cannot easily be determined, since this mutant shows no appreciable competitive disadvantage during growth in mixture with wild type (*14*).

I turn next to a series of phenotypically dependent mutations in the *r* genetic complex, of particular interest because the several mutations are concerned with quantitative variations in a single physiologic mechanism. The analysis is again facilitated because certain of the mutations prove to be reversible.

This series of mutations I discovered only after completing the study of *r* mutation proper, and this fact I attribute partly to improved discernment and partly to the natural tendency when selecting a mutant plaque to choose the most extreme of the visible aberrations. On plating large samples of virus from different clones of T2H, one finds with about equal frequency typical *r* mutants, and a lysis-inhibitor weaker (phenotype *w*) than wild type, but stronger than *r* (which might have been called noninhibitor). If, in turn, one examines clones of *w*, one finds *r* mutants indistinguishable phenotypically from those originating directly from wild type.

In addition to these mutants, which can be obtained at will, I picked up from the stock of T2H a single plaque of aberrant type yielding a new line which is a stronger (phenotype *s*) lysis-inhibitor than wild type. From *s*, exactly as from wild type, one obtains the phenotypes *r* and *w*, and from the latter an additional *r*. (Another phenotype originating from *s*, similar to wild type, was found and analyzed according to the scheme that follows. It proved to differ genotypically from wild type, and can be omitted from discussion without altering the nature of the results.)

This gives us four phenotypes: *s*, wild type, *w*, and *r*, in order of descending potencies of lysis-inhibition. These are distinguishable by type of plaque, as shown in Fig. 4, a, b, c, prepared by photographing platings of mixed pairs of mutants. It will be seen also that lysis-inhibition is phenotypically dominant; that is, where two plaques of different type overlap, the degree of lysis in a part of the overlap is always that of the stronger lysis-inhibitor of the pair. Mixed platings of this kind with known stocks can be used to classify any mutant of the series where the phenotype is in doubt on direct examination.

The mutants whose isolation has been described include two rapid lysers (i.e., noninhibitors) and two weak inhibitors, one of each pair arising from strong and one from wild type, and two additional rapid lysers arising from the two kinds of weak. For the pattern analysis, two independently arising lines of each of these six mutants, together with two

single-particle clones each of strong and wild type, were carried through a series of 25 serial transfers in broth culture in the manner previously found (*14*) to ensure recovery of back-mutant from *r*. These lysates were plated at intervals and examined for the appearance of mutants. At every mutational step, a clone of the new type was isolated, classified as to type of plaque, and tested for host range. No changes in host range were observed anywhere in the series. In every case the mutants of similar origin behaved similarly, and in the cases where two successive mutations were selected during passage, the sequence was clearly evident in that the first type largely disappeared before the third began to show itself. In all cases, too, the changes were step-wise; i.e., platings always showed one or another or a mixture of the previously classified phenotypes.



FIG. 3. Mutational pattern of T2H with respect to type of plaque.

The mutational pattern revealed by this method is shown in Fig. 3, where the frequent mutations, selected by direct examination of small clones, are indicated by broken arrows in the upper part of the scheme, and the slower mutations, unobtainable directly but selected by natural competition during continued growth, are indicated by continuous arrows in the lower part.

The important features of this pattern are the following.

(1) The rapid mutations found are always in the direction of weaker lysis-inhibition.

(2) The mutations selected by serial passage are always in the direction of stronger lysis-inhibition.

(3) Wild type, and phenotype strong, which originated from wild type in an unknown manner, alone among the phenotypes predominate in stable populations. Thus the selection of strong at the expense of wild type, which would be expected according to rule (2), does not occur, but a mutant of strong resembling wild type (omitted from the figure) reverts to strong very readily.

(4) In every case the reversion of mutants from weaker to stronger lysis-inhibition passes through the sequence, and ends in the phenotype, predicted

from their origin. It follows that the four pheno-
typic *r* mutants, and the two phenotypic *w* mutants,
differ genotypically in a manner determined by their
origin.

The pattern shown in Fig. 3 permits the inference
that at least three independent genetic sites, each
subject to two allelic modifications, make up the
complex governing lysis-inhibition (and the pheno-
type analyzed but omitted from the figure requires
one more). The appropriate minimal assumptions
are stated in the form of genetic symbols in brackets
in the figure.

This interpretation is not, of course, the only
conceivable one. But any interpretation in terms of
multiple alleles at a single genetic site seems to me
hopelessly complicated. This would require, for
either half of Fig. 3, four alleles each mutating
preferentially in one or two specified directions.

I want to describe very briefly one more variant
of obscure origin, this time of T6, which demon-
strates rather clearly that mutation rate is geneti-
cally determined. This variant turned up just once
as a very large minority among the progeny of what
was supposed to be a pure stock of T6*r*. Its host
specificity identifies it as an authentic derivative of
T6.

The variant plaques are basically of the lysis-
inhibiting type, like wild type (Fig. 4, d, e), but
are characteristically mottled after the manner of
mixed plaques of *r* and *r*⁺ virus. Nearly every plaque
shows 2 or 3 sectors of rapid lysis, and when the
plaques are picked and replated one always gets
the same mottled plaques in a mixture with about
an equal number of typical *r* (Fig. 4, f). If the
mixed clones are propagated a few times without
repicking, the *r* form quickly wins out. Picking
from the *r* plaques likewise yields pure *r* stocks, and
this *r* mutant differs from that arising from wild
type in not reverting to lysis-inhibitor on serial
passage. Again one is tempted to conclude that the
new genetic factor is not an allele of *r*, but that
factors outside the *r* genetic site determine the rate
of *r* mutation. The alternative is to assume a new
lysis-inhibiting allele of *r* (or a new *r* site), which is
characteristically unstable, and which goes always
to another new allele phenotypically *r*, but charac-
teristically stable or reverting only to unstable.

### Separation of Genetic Factors

We have seen that rate analysis and pattern
analysis of mutations involving independent pheno-
types succeed in answering the primitive questions
of genetics, but that these methods are less satis-
factory when the phenotypes are interdependent.
Fortunately, an additional method of analysis has
recently emerged from the study of what happens
when a single bacterial cell is infected simultane-
ously with two types of virus.

Interest in this kind of experiment dates from the
first quantitative study of mixed infection of bac-

teria, and of interference between bacterial viruses,
published by Delbrück and Luria in 1942 (9). The
most important developments are the mutual-ex-
clusion effect and the depressor effect (7), and the
directed *r* mutation recently reported by Delbrück
(8). Dr. Delbrück is going to describe at this Sym-
posium his new experiments on induced mutation
in the *r* genetic site. His findings, which he has freely
communicated to me, have suggested experiments
along similar lines but yielding somewhat different
results. One of these experiments I want to discuss
from the point of view of its bearing on the prob-
lems we have been considering here.

Recall that from T2H one gets the two host-range
mutants $h^b$ and $h^o$, selected by two different bac-
terial strains resistant to T2H. The mutant $h^o$ acts
on only one, but $h^b$ acts on both, of these strains,
so that we have a typical case of dependent pheno-
types. Rate and pattern analysis suggest, however,
that the two genetic factors may be independent,
in which case the mutant derived from $h^o$ by a sec-
ond mutational step, and acting on both resistant
bacterial strains, has been properly named $h^o h^b$.
The alternative is a third mutant allele, which might
be named $h^{ob}$, at a common *h* locus. Evidently this
alternative can be excluded, if we can somehow
effect a separation of $h^b$ from $h^o$.

Suppose we infect a bacterium with both wild
type ($h^+$) and the virus derived from it by two
successive mutations, our putative $h^o h^b$. If we suc-
ceed in getting out any type that is neither $h^+$ nor
$h^o h^b$—i.e., if we get any induced mutations in the
*h* genetic complex—first, we will have succeeded
in showing that host range is indeed governed by a
complex of independent genetic factors, and second,
we should be able to distinguish the hypotheses $h^{ob}$
and $h^o h^b$ regarding the genetic structure of the
double mutant. If the factors are independent, we
ought to find both $h^b$ and $h^o$ among the mutant
progeny.

In doing this experiment, I chose not wild type,
but $h^+r$, together with $h^o h^b r^+$, for the following rea-
son. When one makes mixed infection with *r* mutant
and corresponding wild type, and then plates out the
infected bacteria before lysis to observe plaques
formed from the centers of mixed infection, one finds
that about 65% of the plaques are mottled, and con-
tain always mixtures of wild type and *r* mutant
(14). I have inferred that the mottled plaques arise
from bacteria in which both types of virus grow.
Some of Dr. Delbrück's experiments throw doubt on
this inference, but at any rate mottling provides an
infallible means of selecting mixed clones. It is only
the mixed clones of this kind, arising from bacteria
simultaneously infected with two or three particles
each of the mutants *h*⁺*r and $h^o h^b r^+$*, that I have
examined in the present experiment. The examina-
tion was made in the following manner.

Ten of the mixed clones were sampled and
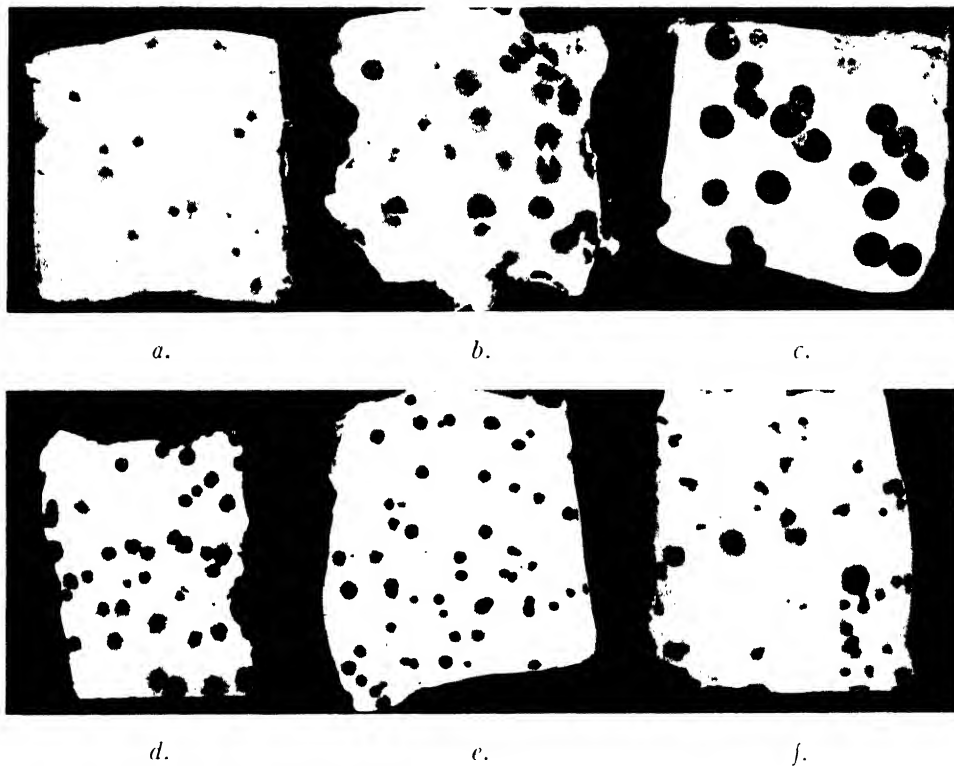plated out again to obtain pure clones each arising

FIG. 4

a. Plaques of strong (s) and 2 or 3 of wild-type T2H, from a plating of the mixture.
b. Plaques of wild-type T2H and weak (w), from a plating of the mixture.
c. Plaques of weak and r mutants of T2H, from a plating of the mixture.
d, e. Plaques of wild-type T6.
f. Plaques of a variant of T6 rapidly mutating to r, and a plaque of the r mutant.

from a single viral particle among those coming from the mixedly infected bacteria. On these plates, the plaques were easily distinguishable as either $r$ mutant or $r^+$. From each of the ten plates, 11 clones each of $r$ and $r^+$ type, totaling 220 in all, were sampled and tested for host range. In these tests, three bacterial strains, previously found adequate to distinguish the phenotypes $h^+$, $h^b$, $h^c$, and $h^ch^b$ (Table 2), were used. The suitability of the sampling and testing methods, and the purity of the original viral stocks, were checked by testing the stocks themselves, and by testing samples from 20 clones each obtained by plating out the stocks. In the case of $h^ch^br^+$, additional tests were made of secondary clones obtained from additional platings of the contents of the primary clones. No variants were found among these check tests, and there was never any doubt as to how the variants arising in the mixedly infected bacteria should be classified. (It is not perfectly clear why $h^ch^b$—and the same is true of $h^b$—remains pure during growth in agar, but not in broth.) Nine of the rarer mutants were retested after plating out again and sampling another clone. These tests invariably confirmed the original classification. The results of all the tests are listed in Table 5.

TABLE 5. INCIDENCE OF PHENOTYPES IN MIXED
CLONES OF $h^+r$ AND $h^bh^cr^+$

Controls: 20 clones each of the two original stocks, and 5 secondary clones from each of 10 plaques of $h^ch^br^+$, yielded no variant types. The stock of $h^ch^br^+$ contained about 1% $r$ mutant.

| Mixed clone | Number of isolates of phenotype | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $h^+r^+$ | $h^br^+$ | $h^cr^+$ | $h^bh^cr^+$ | $h^+r$ | $h^br$ | $h^cr$ | $h^bh^cr$ |
| 1 | 1 | 0 | 6 | 4 | 10 | 0 | 1 | 0 |
| 2 | 0 | 0 | 8 | 3 | 6 | 0 | 5 | 0 |
| 3 | 0 | 0 | 0 | 11 | 5 | 6 | 0 | 0 |
| 4 | 2 | 0 | 6 | 3 | 8 | 0 | 3 | 0 |
| 5 | 0 | 0 | 2 | 9 | 10 | 0 | 1 | 0 |
| 6 | 0 | 0 | 10 | 1 | 6 | 1 | 3 | 1 |
| 7 | 0 | 0 | 11 | 0 | 2 | 0 | 9 | 0 |
| 8 | 0 | 0 | 8 | 3 | 3 | 0 | 8 | 0 |
| 9 | 0 | 0 | 11 | 0 | 11 | 0 | 0 | 0 |
| 10 | 2 | 0 | 3 | 6 | 10 | 0 | 1 | 0 |
| Total | 5 | 0 | 65 | 40 | 71 | 7 | 31 | 1 |
| Per cent | 2 | 0 | 30 | 18 | 32 | 3 | 14 | 0.5 |

The hypothesis of independence leads to the prediction of eight possible phenotypes. All but one of these are found among the progeny of the mixedly infecting viruses. The single exception is $h^br^+$; though its rapidly lysing counterpart $h^br$ is also rare, being isolated only seven times from two of the ten mixed clones. The rarity of the $h^b$ phenotype can be attributed to the fact previously brought out, and trivial with respect to the purposes of this experiment, that pure stocks of this type grow very poorly on sensitive bacteria. That selection against

this factor occurred during the growth of the mixed clones is suggested also by the fact that only 21% of the isolates contain $h^b$ in any combination, although 65% contain some factor derived from $h^ch^br^+$.

Apart from the above considerations, the frequent occurrence of $h^cr^+$, which on statistical grounds must arise largely by loss of $h^b$ from $h^ch^br^+$, and of $h^cr$, which must arise largely by transmission of $h^c$ from $h^ch^br^+$ to $h^+r$, seems to point conclusively to the independence of the factors $h^b$ and $h^c$. I am here employing a new criterion of independence, to be sure, but it may be tentatively assumed to include the former one, for it is difficult to imagine a model permitting independent transmission of genetic factors that could not independently undergo spontaneous mutations.

The other pair of rare types, $h^+r^+$ and $h^ch^br$, are interesting in that they immediately suggest a correlation between the low frequency of loss of $r$ by $h^+r$, and of gain of $r$ by $h^ch^br^+$. It should be mentioned that the single instance of $h^ch^br$ found is the only one among the new types that could easily have arisen by spontaneous mutation. In fact, this experiment by itself does not yield convincing evidence for the transmission of the $r$ genetic factor, though the mutant $h^+r^+$, isolated five times from three of the ten mixed clones, strongly suggests that transmission occurs.

In discussing this experiment I have tacitly assumed the transmission of mutant factors to explain induced mutations, the wild-type allele being regarded as a material deficiency. The converse formulation is of course equivalent genetically, mutants being considered deficient with respect to a specific wild-type factor corresponding to each mutant found. A third alternative exists: the possibility that a given mutation requires both a loss and gain (or internal rearrangement) of genetic material. Insofar as mutations, both spontaneous and induced, probably occur during synthesis of the viral particles, it is not evident that these three (or four) hypotheses lead to different statistical predictions, and they may be considered equivalent for the purposes of this experiment.

These data, in the absence of further experiments, and particularly in view of the complication that some of the mutants compete poorly during growth, need not be discussed further here. It is evident, however, that suitably modified experiments of this kind may provide a means of analyzing transmission frequencies, for purposes quite apart from the qualitative test of independence of genetic factors.

Experiments along the same lines provide a clue, too, to a possible mode of origin of new viral types. Rather frequently one finds, in presumably pure viral stocks, host-range variants that correspond neither to any wild-type virus in the laboratory nor to identifiable spontaneous mutants. At the same time, the new types are usually too closely related,

serologically and otherwise, to the well-known types to be accounted for as foreign contaminants. In one instance I got such a variant in a stock of T2K*r* (*14*) which had obviously been contaminated by an *r*⁺ virus. This variant I have since been able to obtain, among other new types, by experimentally infecting bacteria with a mixture of T2K*r*⁺ and T2H*r*. It is easy to imagine that it arose in the first place following contamination of T2K*r* with T2H, and that this sort of hybridization may serve in nature to multiply the seemingly unlimited array of existing types of virus.

### Mutation in Animal and Plant Viruses

Evidence for mutations in animal and plant viruses is to a high degree circumstantial. The classical example of experimental alteration of heritable characteristics in a virus is the adaptation of rabies virus from the dog to the central nervous system of the rabbit by Pasteur. Burnet and Stone (*6*) have recently described an adaptation of human influenza virus to the allantoic cavity of the chick embryo. This adaptation occurs during serial passage by the amniotic route, but can be prevented by transferring sufficiently small inocula taken at suitable times. From these and many other examples (*11*), the generalization can be drawn that such adaptations result in altered host or tissue specificity of the virus without appreciable change in antigenic character. Apart from their considerable practical importance, there is no reason to suppose that these adaptations differ in any way from the host-range mutations of bacterial viruses.

Among the plant viruses, several instances of mutation, mostly recognized by altered character of lesions, are known or suspected (*2*). Parallel mutations in closely related wild types of tobacco mosaic virus suggest the existence of independent genetic factors, though the postulated mutational patterns have not so far been mapped out experimentally (*2, 23*). This suggestion is nevertheless important in indicating that genetic complexity is not limited to the structurally complex bacterial viruses I have referred to in this paper.

Reverse mutations have generally not been demonstrated in plant or animal viruses (*2, 11*), though exceptions to this rule suggest that the failure may be due solely to technical difficulties. The mutations of plant viruses are often in the direction of lessened virulence (*25*), and the ability of the mutant types to suppress the growth of the parent virus probably explains both the outgrowth of the mutant and its "fixed" character. When the mutant is of slow-moving type, or when it is transferred to a new host plant, apparent reversion sometimes occurs (*2*).

Recognizable mutations in mosaic virus are not accompanied by appreciable chemical, crystallographic, or serological changes (*30, 26*). Changes in isoelectric point and electrophoretic mobility have, however, been reported (*26*). More distantly

related wild types show differences in amino-acid composition, antigenic specificity, and crystal habit (*2, 30*). The constancy of antigenic character during single mutational steps seems to be without exception among plant, animal, and bacterial viruses.

### Effects of Radiation

Gowen (*12*) has reported an increase over the spontaneous rate of mutation of ordinary tobacco mosaic virus to aucuba as a result of X-irradiation of dried viral preparations. The phenotypic reverse mutation was affected in the same way. Kausche and Stubbe (*19*), on the other hand, could not obtain significant effects by irradiating purified mosaic virus, but reported large increases in visible mutations by irradiation of inoculated leaves. Luria, and Luria and Latarjet (personal communication), have tried repeatedly to induce host-range mutations in the bacterial viruses T1 and T2 by ultraviolet irradiation of the isolated viruses, without observing significant increases over the spontaneous frequencies.

The difficulties attending the study of mutation in plant and animal viruses, mentioned in the introduction to this paper, are even greater when radiation is employed. If, for instance, one were examining lesions arising from a mosaic-virus preparation containing a certain proportion of mutant particles, irradiation might under certain circumstances increase the frequency of detection of mutants without altering in the least their actual proportion, merely by reducing the average number of active particles producing the lesions. Salaman (*25*) has found that certain plant viruses, previously classified as separate types, are actually mixtures of two other types. He has shown also that artificial mixtures of two viruses may behave like either one of the component types, depending on the proportion in the mixture. An "activation effect" of irradiation on the infectivity of tobacco mosaic virus (*18*) implies the existence of other complications of unknown nature. It is doubtful whether significant rates of mutation, either spontaneous or induced, in plant or animal viruses, can be measured by present methods.

The limitations of available methods of studying viral mutations are mentioned again here because it seems important to determine rigorously whether or not mutations, identical with the spontaneous ones, can be brought about by irradiation of isolated viruses, and to compare the effects on viruses in the presence of cells or cell products. Bacterial viruses should furnish unique materials for this purpose, and the data might have an important bearing on the problem of the nature of mutation.

### Discussion and Summary

The viruses form unique objects for genetic study because of their relatively simple structure, and

their partial independence of the cells in which they grow. What has been learned about them, in general terms, is that they exhibit a remarkably complicated genetic behavior, and that they undergo mutation principally or exclusively during their intracellular existence. Although the results detailed in this paper refer to one of the larger bacterial viruses, there is some reason to believe that the general implications hold good also for structurally simpler bacterial and plant viruses.

In the bacterial virus T2H two classes of mutation have been studied, one affecting host range, the other affecting type of plaque. Each of these characters is determined by several loci of independent mutation. At each mutational site, there seems to be a single structure subject to reversible alternation between only two alleles. There is not yet any evidence either for multiple allelism, for coupled mutation, or for mutation by loss.

Failure to find multiple alleles might be attributed simply to the kind of methods used. Analysis by mutations alone undoubtedly reveals the finer structure of genetic material, as opposed to analysis by crossing over, which reveals the coarser (29). It is perhaps not certain that multiple alleles that would meet the requirements of the definitions used here exist in any genetic material.

Failure to find coupled mutations, or mutation by loss, might be interpreted as the one indication of simplicity of genetic structure for the virus. If the generalization should emerge that the viral particle carries few or no dispensable genetic factors, this would clearly differentiate viruses from cells.

In the genetic complex determining plaque type there are evidently a number of genetic factors exerting their influence through what is, superficially at least, a single physiologic mechanism: lysis-inhibition. These factors mutate independently, and the question arises whether their mutual relation has a different stuctural basis from the relation between these factors on the one hand, and the physiologically unrelated factors governing host range on the other. So far the available methods of analysis have not given any indication of different degrees of structural relationship, to correspond with the alleles, crossover units, and linkage groups of conventional genetics, or with the unit factors and linkage groups (?) of bacteria (Luria, this Symposium). It is not likely that this difference is due solely to the limitations of method, and further study may well reveal new relationships.

A new and promising approach to questions of this kind is by means of the mixed-infection experiments in which one observes an apparent segregation of genetic factors. Whether this phenomenon is related to crossing over, and indeed whether it implies actual exchange of genetic material at all, are pressing and important questions. But whatever the mechanism responsible for the impressed mutations, it is probable that the purely genetic study of their patterns will yield valuable clues to the

genetic structure of viruses. It is interesting that so far the use of this method has only served to confirm inferences drawn from the study of patterns of spontaneous mutation.

If it may be assumed that there is anything in common in the transformation of pneumococcal types (1), the fibroma-myxoma transformation (4), the induced mutations with respect to lysis-inhibition in bacterial viruses (Delbrück, this Symposium), and the directed mutation with respect to host range described in this paper, it now appears for the first time that a biological phenomenon of general importance is involved. In the bacterial virus, three genetic factors chosen more or less at random for the test all appear to be transmissible from one viral particle to another. In view of the generality of this phenomenon, it will be surprising not to find that some counterpart to it already exists among the more familiar genetic mechanisms. Among the viruses, it is clear that it provides an additional mechanism for the production of new biotypes.

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## DISCUSSION

DOERMANN: After Dr. Hershey's description and interpretation of the r and r+ variants of the viruses T2, T4, and T6, a few remarks about our experiments with these viruses may be appropriate. First, however, I would like to draw your attention to the accompanying chart, which graphically shows what we mean by the term "lysis-inhibition."

The two curves represent the turbidities of two bacterial cultures over a period of time, in which time zero is the time of addition of virus, in both cases to a titer of about five times the titer of the bacterial culture. It is seen that the turbidity drops immediately after the addition of virus and within a few minutes rises again. This initial drop is not due to lysis, as has been shown by plaque counts. It is apparently associated with some change in the light-scattering properties of the bacteria, which

seems to be related to the adsorption of the virus or to its penetration into the cell.

The second drop seen in both experiments occurs at about 23 minutes after infection. That this is the beginning of lysis is known from the fact that there is a sudden rise in phage titer at this time. The difference between the two curves appears in this drop. With T4r, lysis is almost complete and cultures appear clear as early as 60 minutes after infection. In the case of T4r+, however, the turbidity drops very little, and even as late as 200 minutes after
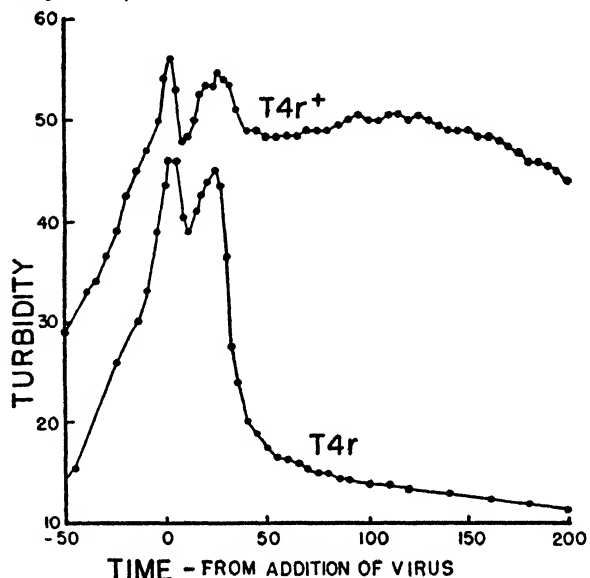


Progressive turbidity of cultures of B multiply-infected with T4r and T4r+. The curve representing T4r+ infection has been scaled up ten units on the vertical axis.

infection the culture is very turbid. Complete clearing takes place after several more hours of incubation.

Our experiments centered around the problem of the ultimate cause of lysis inhibition, in order that we might discover what the basic difference is between an r and an r+ virus. If an infected culture is diluted before the end of the latent period, lysis occurs similarly and without delay in both r- and r+-infected bacteria, as shown by plaque counts. This is the result typically found in the one-step growth experiments. Contrasting such a result with that found in moderately turbid cultures, like those represented in the figure, suggests that an inhibitor is produced, but that it is not concentrated enough to be inhibitory in the diluted cultures.

We tested this hypothesis by making a standard inhibited culture using T2r+ on B, centrifuging out the inhibited bacteria, and testing the supernatant for inhibitory effect on diluted suspensions of B infected with either T4r or T4r+. The T4 titer of the diluted suspensions was assayed at short intervals with B/2 in order to determine the time of lysis of the bacteria. The result was that the su-

pernatant delayed bursting of T4$r^+$-infected bacteria but not those infected with T4$r$. In the control suspensions diluted in broth, lysis began at 23 minutes as expected.

Several conclusions can be drawn from the experiment described. The supernatant of a centrifuged inhibited lysate does contain an inhibitor. It can also be concluded that an $r^+$ virus must be present in the bacterium to be lysed in order that inhibition may be effected.

Subsequent experiments have shown that the inhibitory principle is present in $r^+$ lysates that have cleared completely, as well as in the culture at the time of inhibition.

The next problem was to learn some of the properties of the inhibiting agent. Since the lysate contained both inhibitor and high-titer virus, several experiments were made to see whether the virus itself might be the inhibitor. Of the experiments described below, some were performed using T2$r^+$ lysates and some with T6$r^+$ lysates. The inhibitory action of these is similar.

One procedure tried for separating the virus from the inhibitor was to adsorb the virus on B in the hope of leaving behind some of the inhibitor. The virus titer was reduced about a hundredfold, but the inhibitor action was similarly diminished. The counterpart of this experiment was to try to adsorb out the inhibitor without removing the virus. The bacterial strain B/6 is known not to adsorb T6, and it was used as an adsorbent. In an exposure to B/6 similar to that used in the previous experiment with B, the virus titer was not reduced significantly, but neither was the inhibiting power of the lysate altered.

We also tried to remove the virus and leave the inhibitor by inactivating the virus with specific antivirus serum. In this manner the virus titer was lowered by a factor of $10^4$, but the inhibitor was also lost.

Two other methods were also tried for separating the virus from the inhibitor. One was high-speed centrifugation. With such a technique the virus titer was cut down by a factor of 20. The inhibitory action was similarly reduced. The other procedure tested was dialysis through cellophane. Neither the virus nor the inhibitor passed through the cellophane.

In summary, one can conclude from the experiments described that the inhibitor is either the virus itself or some substance closely bound to the virus, since:

(1) Lysates of $r^+$ viruses are effective as inhibitors.

(2) The virus and the inhibitor have the following properties in common: (a) both are adsorbed on B and neither is adsorbed on a bacterial strain resistant to the virus; (b) both are inactivated by the same specific antiserum; (c) both are thrown down by high-speed centrifugation; and (d) neither will pass through a cellophane membrane.

LATARJET: In connection with Dr. Hershey's conclusion that spontaneous mutations in phages occur only during their life inside the bacterium, I would point out the following observation made in the course of experiments recently carried on with Dr. Luria. Irradiating diluted stocks of free phage T2 with ultraviolet 2537 A, doses going up to about 1000 ergs $\times$ mm.$^{-2}$, we have been unable so far to induce mutations.

SONNEBORN: If $r$ and $w$ are mutations at different sites, it would be expected that back-mutations of $w$ to wild type could precede back-mutations of $r$ to $w$ in the $r$ mutants that have arisen from the $w$ mutant. In such a case, since the prior mutation of $w$ to wild type would give no phenotypic effect, one would have an apparent back-mutation of $r$ directly to wild type without going first to the $w$ phenotype. It would seem that a demonstration of this possibility is needed to supplement the evidence that $w$ and $r$ are at different sites.

This possibility further raises a question concerning the use of the criterion of two-step reversion in distinguishing "multiple alleles" from "independent loci." In higher organisms, multiple mutants are often less vigorous than single mutants. This raises the question whether, in cases comparable to the one cited in the preceding paragraph, lines of descent in which $w$ had reverted to wild type might outgrow those in which such a reverse-mutation had not taken place. This would then increase the chance of finding apparent single-step reversions in cases of two-step mutants; but if the mechanism is the one here suggested this would not constitute evidence for "allelism." Although this does not seem to happen in the example cited, it might well take place in some other series of multiple mutants and should therefore be recognized as a possible source of misleading evidence in the study of allelism and independent sites.

ZAMENHOF: The problem of chemical differences between two mutant viruses is of extreme importance. There is a paper by Pfankuch in which the investigation of chemical differences between two strains of tobacco mosiac viruses is described, one strain being a direct mutant of the other—that is, obtained in a single mutational step. This investigation on crystalline viruses revealed that there was no difference in amino-acid content, but a significant difference in the nucleic acids of these two strains; namely, there was a difference in phosphorus content and a difference in migration velocities in the electrophoretic cell. However, one has to be careful with such results, because electrophoretic study of thymus nucleic acid reveals one very sharp peak, suggesting one single substance, although there are thousands of different genes in a thymus cell nucleus, and these genes may differ more than the above-mentioned strains. It may be, therefore, that our tools are not yet refined enough to detect these differences, which undoubtedly must exist.

# INDUCED MUTATIONS AND SPECIATION IN FUNGI

A. HOLLAENDER and C. W. EMMONS

The ready production of a wide variety of mutants in fungi by ultraviolet radiation (7), X-rays (13), and neutrons (16) has raised a number of problems of special interest.

(1) Some of the mutants produced by radiation resemble closely certain naturally occurring fungi which have been generally accepted as separate "species." Other mutants are of a type which could be classified as new "species" if one did not know that they had originated from certain fungus cultures.

(2) It has been possible to produce mutants in certain fungi by irradiation with wave lengths in the ultraviolet spectrum that are present in sufficient intensity in sunlight coming to the earth's surface that they may be a factor in the production of mutations in nature.

Variability of a kind not susceptible to traditional genetical analysis is frequently observed in the *Fungi imperfecti* and is often an interfering factor in their classification. It is highly desirable to establish the type and frequency of such variation. In the case of a number of fungi and yeasts, it has been possible to analyze these variations genetically (5, 19, 15). A report of a series of remarkable mutations appearing spontaneously in an old culture of the pathogenic fungus *Microsporum gypseum* concluded with the hypothesis, "many of the dermatophytes now known as species are only varieties of a single unstable species" (6).

It would lead too far to go into details of the work on the effect of ultraviolet radiation here, especially since it has been reviewed very well by Dr. Laterjet in the paper by Demerec and Laterjet, and has been published in a previous Symposium (10).

The first part of our paper reviews work with *Trichophyton mentagrophytes*. This fungus is superior to other fungi we have used in radiation and mutation studies, because of the relative ease with which the experienced mycologist can distinguish between contaminants and mutants. In general, this fungus is not airborne and we have not been able to pick it up as a laboratory contaminant. The spores of Neurospora, *Aspergillus terreus,* and *Penicillium notatum* (also used in radiation studies) are less satisfactory because they are found occasionally as contaminants in the air of most laboratories. These and other contaminants are demonstrated readily if effective testing methods, which have been developed during the last few years for the sampling of airborne microorganisms, are used.

The frequent observation of variations in many fungi and the many published reports of so-called new "species" have given special interest to the ready production of such variation by ultraviolet radiation and by some penetrating radiations.

In studies on the effects of ultraviolet radiation and X-rays on fungi we have observed: (1) lethal effects, (2) temporary physiological changes, and (3) up to 40% to 80% mutants in the surviving spores (7, 13, 11). Representative mutants, selected from several hundred mutants of *Trichophyton mentagrophytes* induced by ultraviolet radiation, have now been carried in culture for six years. They still exhibit the characteristic features that led to their selection (10).

The identification of these forms as mutants is based on their sudden appearance and permanence, on the fact that they are actually dermatophytes, and on evidence that they were not contaminants isolated in culture by chance from airborne spores. The permanence of the changes is illustrated by the period (6 years) during which they have been kept under observation and frequently transferred (more than 20 times). The mutants are certainly dermatophytes. The conidia and macroconidia, although abnormal in size or shape in some mutants, are

TABLE 1. NUMBERS AND TYPES OF MUTATIONS OBTAINED IN A TYPICAL EXPERIMENT, WITH ASPERGILLUS TERREUS, USING 2650 A

| Colony Type | Control | R-1 | R-2 | R-3 | R-4 | R-5 | R-6 | R-7 | R-8 | R-9 | R-10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Normal appearing—red exudate | — | 1 | — | — | 1 | — | 1 | — | — | 1 | — |
| 2. Very woolly—dark diffuse pigment | — | — | 1 | — | — | — | 1 | — | — | — | 1 |
| 3. Yellow woolly | — | 1 | — | 2 | 1 | — | — | — | — | — | 1 |
| 4. White woolly | — | — | — | 1 | — | — | — | — | 1 | — | — |
| 5. Slow-growing—small | — | — | 1 | 1 | — | — | — | — | — | — | — |
| 6. Limited colony—uniform appearing | — | 1 | — | 1 | 3 | — | 1 | — | — | 1 | — |
| 7. Limited colony—raised, late sporing | — | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8. Leathery | — | — | — | — | — | — | — | — | — | 1 | — |
| 9. Erupted | — | — | — | 1 | — | 1 | — | — | — | — | — |
| 10. White conidial heads | — | — | — | 1 | — | — | — | — | — | — | — |

easily identified as those of Trichophyton. Conclusive proof that the mutants are dermatophytes is provided by the demonstration that they retain their pathogenicity for animals. The parent strain produces in guinea pigs a severe dermatophytosis (ringworm), and the mutants tested also produce characteristic skin lesions, although in most cases the severity of the lesion is reduced. It is equally clear that these mutants were not contaminants. During the course of the radiation experiments 5000 nonirradiated conidia were picked from agar plates and cultures. No mutant was observed among the 5000 colonies resulting. These 5000 control conidia were young spores of the same age as those irradiated. It was shown, however, that when old nonirradiated conidia were selected and cultured mutants (in some cases of the same type as the induced mutants) appeared spontaneously. The mutants were not other strains of dermatophytes appearing as contaminants. In most cases they were unlike any other strains kept in this laboratory, and, as previously mentioned, dermatophytes have never been detected as airborne contaminants in this laboratory.

In about half the experiments, plating methods were used to insure purity of the strain. In the remaining experiments all conidia irradiated were the progeny of a single conidium picked under the microscope. Irrespective of the method of purification of the strain, all colonies derived from young nonirradiated conidia were like the parent, while a high percentage of colonies derived from conidia surviving irradiation differed from the parent in obvious and permanent respects. In these experiments no attempt was made to detect physiological mutants not apparent by simple examination of colonies.

The fungus used in these experiments was a strain of the variable species *Trichophyton mentagrophytes* similar to the variety described by Sabouraud as *T. gypseum asteroides* (Fig. 1). On acid dextrose agar (modified Sabouraud's agar) it produces a flat colony which is cream-colored at the center, fading to white at the periphery. This aerial mycelium covers reddish-brown hyphae, which grow in the substratum and impart a reddish color to the reverse side of the colony. The surface is granular, owing to the production of large numbers of conidia, and is marked by radial strands or ridges which give the colony an asteroid appearance. The fungus produces one-celled subspherical conidia, clavate one-to-several-celled macroconidia, and abortive ascogonia, all of which are typical of Trichophyton.

When the conidia of this fungus were exposed to sufficient ultraviolet radiation (2537 or 2650 A) so that 90 to 99% of them were killed, a high percentage (up to 40%) of the surviving conidia gave rise to colonies strikingly different from the parent. As previously pointed out, these mutations were permanent. Some of the mutants resembled other dermatophytes generally recognized as distinct species, and many of the others bore such distinctive characteristics that, were their origin unknown, they probably would be assigned by a taxonomist to new species. Some representative strains have been described in considerable detail in another publication (*8*). Only a very brief description will be given here.

The mutant shown in Fig. 2 resembles the dermatophyte usually designated *Trichophyton interdigitale*. It produces more aerial mycelium than the parent strain, and there is a corresponding reduction in pigmentation and in the number of spores produced. There is also a decrease in virulence for guinea pigs. In some other mutants the reduction in sporulation and pigmentation and the increase in aerial mycelium are greater and the mutants closely simulate the so-called pleomorphic forms of the dermatophytes. We present the experimental production of mutants of this type as evidence supporting the hypothesis (accepted by many mycologists) that the many varieties of *Trichophyton gypseum* and related forms are merely varieties of one variable species, for which we prefer the earliest valid name, *T. mentagrophytes*.

In the mutant shown in Fig. 3 the surface is more finely granular than in the parent because of the smaller aggregations of conidia. The peripheral hyphae extend in a loose radiating fringe. In microscopic morphology, pigmentation, and virulence for animals this mutant resembles the parent, but is definitely and permanently differentiated from it by colony morphology.

The mutant shown in Fig. 4 is typical of many produced. There is a decrease in aerial growth, an increase in the ratio of conidia to sterile aerial hyphae, and profuse production of an intense purplish-red pigment, which is produced in the hyphae growing on and in the substratum and is very apparent through the white aerial hyphae and conidia. Mutants of this general type, when tested on guinea pigs, were found to be as virulent as the parent.

In mutants of the type shown in Fig. 5 a reduction in aerial hyphae and conidia and in size of colony, and an increase in the amount of reddish pigment produced, have proceeded to a point where the fungus bears a striking resemblance to the well-known species *Trichophyton violaceum* (Fig. 6). The colony has a wrinkled surface, covered in part with a sparse white bloom consisting of short hyphae and conidia. The color is reddish violet. This mutant produces more spores than *T. violaceum* and the pigment contains less blue than in that species. Its virulence for animals is less than that of the strain from which it was derived, but greater than in the case of *T. violaceum*, and the type of hair invasion is ectrothrix rather than endothrix. This mutant is not *T. violaceum* but there

is a sufficient resemblance to stimulate speculation about a possible mutational origin for *T. violaceum*, which, in the United States, is rare and apparently somewhat sporadic in distribution. The variability among strains of *T. violaceum* of different origin, and the instability of a single strain (particularly its loss of pigment production) when kept over a period of months or years in the laboratory, are known to all who are well acquainted with this species. The instability of this species is compatible with a mutational origin and parallels the variability of other dermatophytes.

After completion of the study on Trichophyton we had an opportunity to study a number of other fungi, especially *Aspergillus terreus* (11, 17) and *Penicillium notatum* (13). With both of these organisms we have had invariably the same experience as with Trichophyton. Table 1 gives the number and types of mutants obtained in a typical experiment using 2650 A on *Aspergillus terreus*. Many

TABLE 2. NUMBERS AND TYPES OF MUTATIONS OBTAINED IN A
TYPICAL EXPERIMENT (EXP. F. 25) USING 3130 A
(*Aspergillus terreus*)

| Run | Time of Exposure | Rel. Energy (ergs) | No. Isolated | No. Mut. | % Mut. |
|-----|------------------|--------------------|--------------|----------|--------|
| 1 | 0 | .0 | 43 | 0 | 0 |
| 2 | 1 hour | $1.2 \times 10^5$ | 93 | 0 | 0 |
| 3 | 2 hours | $2.3 \times 10^5$ | 83 | 6 | 7.2 |
| 4 | 3 hours | $3.4 \times 10^5$ | 73 | 6 | 8.2 |
| 5 | $4\frac{1}{2}$ hours | $4.8 \times 10^5$ | 110 | 1 | .92 |

of the *Aspergillus terreus* mutants were deficiency types similar to the ones reported by Beadle and Tatum (2) for Neurospora. The chemicals that these organisms were unable to produce could, if supplied to the cultures, make them appear normal; for instance, Vitamin $B_1$, nitrogen in the form of ammonia, etc.

We have extended our previous studies of the effects of monochromatic radiation between 2180 and 2967 A to more detailed investigation of 2967 and 3130 A. These wave lengths appeared to us especially interesting because they are present in the biologically effective part of sunlight reaching the surface of the earth—that is, 2900 to 3150 A. (The term "biologically effective" when used in radiation work refers to the erythema, and Vitamin-D producing, portion of the spectrum. The long ultraviolet and visible part of the spectrum also affect biological as well as other functions; for instance, the lethal effect of the long ultraviolet and photosynthetic activity of visible light.) The two wave lengths (2967 and 3130) that were tested were found to be effective in the production of mutations in *Aspergillus terreus*. Table 2 gives the results of a typical test made with *Aspergillus terreus*. The energies that will produce mutations at these wave lengths are ten to fifty times greater than those found for 2650 A. These long wave

lengths not only are less effective in producing mutations but also do not produce rates over 10%, whereas 2650 A will produce mutations up to 50% of the surviving spores. That 2967 and 3130 are capable of producing mutations is rather interesting, because these wave lengths are included in the biologically effective part of sunlight.

The emission of biologically effective sunlight at 3150 A and shorter, reaching the surface of the earth, has been investigated carefully for a number of years by Dr. Coblentz of the National Bureau of Standards. The values given below have been taken from Coblentz's Ives Medal lecture before the Optical Society (3). These measurements were made with a photo cell sensitive only for wave lengths shorter than 3150 A, in combination with a trigger-type amplifying unit. Fig. 7 gives the diurnal variation of total and biologically effective sunlight on a clear October day in Washington, D.C. Fig. 8 gives the average per month for a five-year period of the biologically effective ultraviolet solar and sky radiation incident on a horizontal plane in Washington, D.C.

The biologically effective radiation on an average bright and sunny day in June amounts to about 2000 milliwatt seconds per cm.². It requires approximately 230 milliwatt seconds per cm.² to produce a 6% mutation rate in the 10% of spores of *Aspergillus terreus* surviving 2967 A radiation. It should thus be possible in about one hour's exposure to bright sunlight around noon in June to produce in this fungus a significant increase in mutation rate.

We will now describe in greater detail the technique used in quantitatively irradiating fungus spores with ultraviolet radiation. We are doing this especially since the technique used with sunlight is very similar to the technique used with 2537 A radiation. There seems to be a feeling, judging from the expression of many of the members of this Symposium, that the techniques of quantitatively irradiating fungus spores with ultraviolet are difficult and involved. You will see from the following description that this is not so.

A typical experiment follows. Spores of a standard *Aspergillus terreus* culture grown for 14 days on potato-dextrose-carrot-agar slants are washed off with a physiological salt solution containing 1 to 100,000 sodium lauryl sulfate, shaken thoroughly, filtered through absorbent cotton, centrifuged, the supernatant discarded, the precipitate resuspended in a saline sodium lauryl sulfate solution, shaken vigorously and again filtered through a heavy layer of absorbent cotton. This is diluted 1/100 with a saline sodium lauryl sulfate solution and 10 cc. are placed carefully in a round-bottom flask made of highly transparent fused quartz. Another 10 cc. of the spore suspension are placed in a pyrex round-bottom or Erlenmeyer flask, covered with amber cellophane, which absorbs practically all radiation
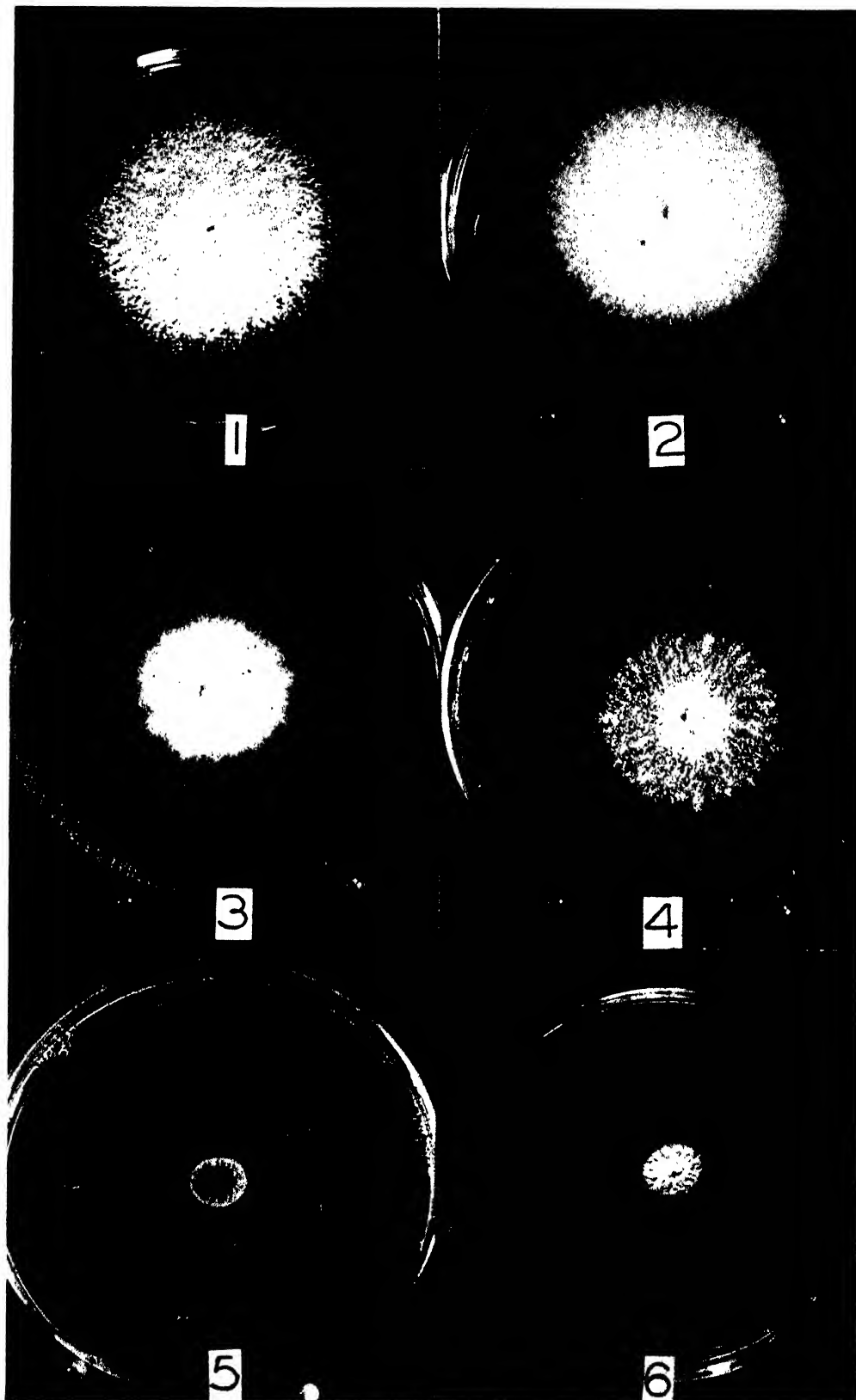
FIG. 1. *Trichophyton mentagrophytes*, parent strain.
FIGS. 2-5. Mutants derived from spores surviving ultraviolet radiation (2650 A).
FIG. 6. *Trichophyton violaceum*.

shorter than 4000 A. Both suspensions are slowly rotated at an oblique angle in such a manner that none of the liquid touches the upper ⅓ of the flask. The liquid, which is kept in movement by the rotating flask, presents continuously a fresh surface to the radiation. This method increases the probability that all of the spores are irradiated uniformly from all sides and that no spores are protected by other spores or by pieces of mycelium that might be present in the suspension. These precautions are

liminary experiments with *Aspergillus terreus* that unobstructed Washington sunlight on a clear, bright June day is able to produce a mutation rate up to 6% in *Aspergillus terreus* spores.

The use of sunlight for routine mutation production is not recommended, since sunlight reaching the earth's surface is at best an unreliable source of ultraviolet radiation; and, as a matter of fact, in heavily industrialized areas or in most big cities it is hardly available as an ultraviolet source.
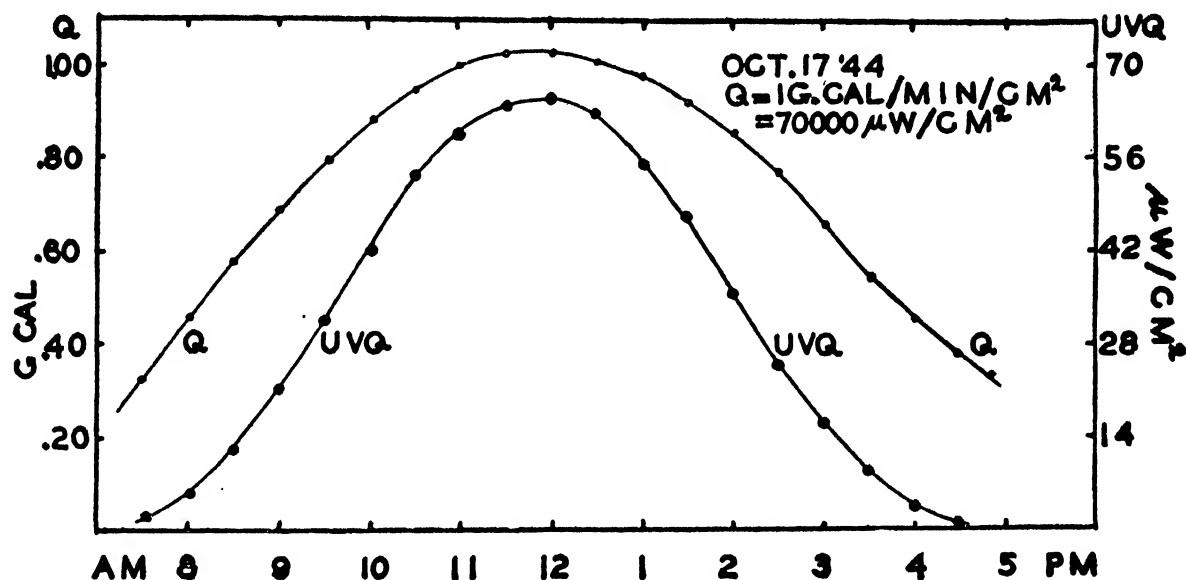


FIG. 7. Comparison of the diurnal variation in the total, Q, of all wave lengths and of the biologically effective ultraviolet component, UVQ, radiation from sun and the whole sky incident in a horizontal plane on a clear day in Washington, D.C. (Taken from Coblentz, J. Opt. Soc. Amer. 36: 74, 1946.)

probably not so important when working with sunlight as when working with 2537 A, because of the greater penetration of the 2900-3150 A region into the spores. The rotating flasks are turned so that they receive the fullest direct sunlight and cooled with a strong blast of air so that their temperature is not raised above the temperature of Washington air. Usually such experiments are started by 10:00 a.m. and samples removed at 11:00 a.m., 12:00 m., 12:20, 12:40, 1:00, 1:30, 2:00, 3:00, and 4:00 p.m. Proper dilutions are plated out on potato-dextrose agar. Survival ratios are determined after 72 hours of incubation, and 50 to 100 single colonies are picked and transferred to potato-dextrose-agar slants. Mutations are picked by visual inspection. The survival-ratio curves indicate that we have not only the effect of the ultraviolet shorter than 3150 A but also an effect of the long ultraviolet, as well as possibly of the infrared. Under laboratory conditions we usually have not been able to observe mutation production by wave lengths longer than about 3150. The effectiveness of wave length 3300 is still in doubt.

In any case, we have been able to observe in pre-

If we consider the abundance of sunlight during the summer months in a cornfield, or the bright sunlight in the tropics, it is not at all surprising that we have a wide variation of types of fungus appearing under natural conditions, particularly in the case of plant pathogens. Some of these mutations may very well be better adapted to the surrounding conditions than the original cultures.

Natural conditions may not always be ideal for the effective irradiation of fungus spores. Biologically effective ultraviolet has a rather low penetrating power, and thus only spores that are not protected by other spores or other materials will receive sufficient radiation. But, in spite of this, a large number of spores that are located on the immediate surface will receive enough radiation to be readily affected. In the study of the ecological distribution and the appearance of natural mutations of fungi, these points will have to be taken into consideration. It is important to keep in mind, however, that ultraviolet is only one of several factors that may affect the mutation rate. For instance, cultures kept in the dark for long periods show increased mutation rates.

We might very well ask: if sunlight can serve

as a source of mutation-producing radiation, why don't we have more somatic mutations in tissues of plants and animals? Probably most of the mutated cells are "deficient" cells, in the sense that they are not so readily adaptable to the surroundings as the

tissue. It may also be difficult for the radiation to penetrate to the proliferating tissues.

However, there are other points which are of interest in connection with this problem. The 3600 A region, which is very intense in sunlight, has, as
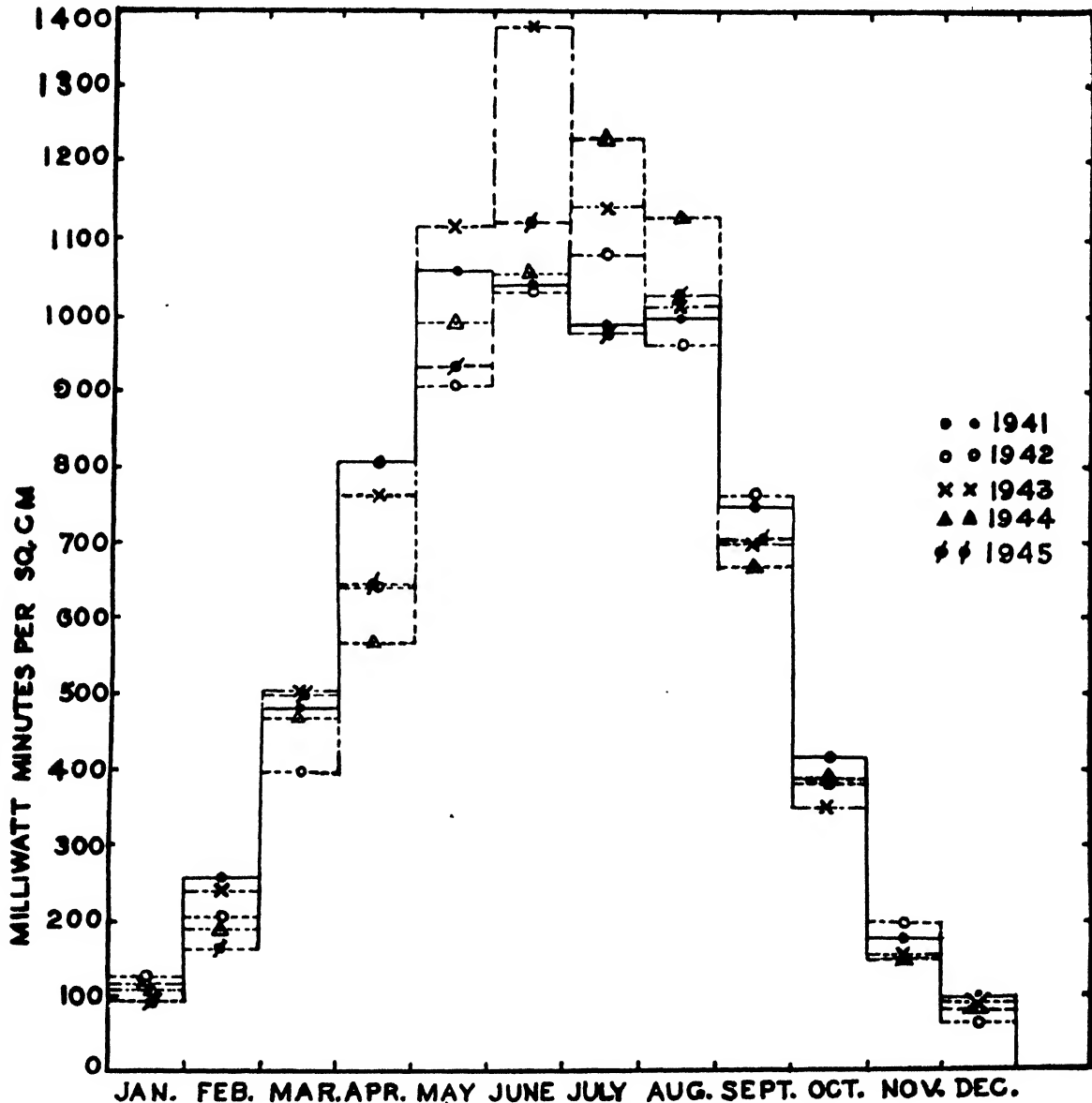


Fig. 8. Monthly totals of biologically effective ultraviolet solar and sky radiation in milliwatt minutes per square centimeter on a horizontal plane in Washington, D.C., February 1, 1941, to September, 1945. (Taken from Coblentz, J. Opt. Soc. Amer. 36: 75, 1946.)

cells from which they originated. Of course, there might occasionally be mutated cells that are better adapted to the surroundings which will survive. We have no information about how often such "adapted" mutations will appear. Somatic mutations may also be obscured by the diploidy of the

we have mentioned, no mutation effect, but this wave-length range has a very strong physiological effect in increasing the normal lag phase of the organism as much as tenfold, as found with *E. coli* (see Fig. 9). (9) Further, organisms after irradiation with this wave length are much more sensitive

to adverse conditions. For instance, a balanced salt solution which will not affect a normal organism will somewhat affect an organism irradiated with 2650 A, and will be fatal to an organism irradiated with 3650 A (see Fig. 10). (9)

Organisms that have survived short-ultraviolet radiation are more sensitive to heat than unirradiated cells, as Curran and Evans (4) have shown for bacterial spores, and Anderson and Duggar (1) for yeast. There is good indication that these phenomena, observed with bacteria and yeast, may also take place with other cells.
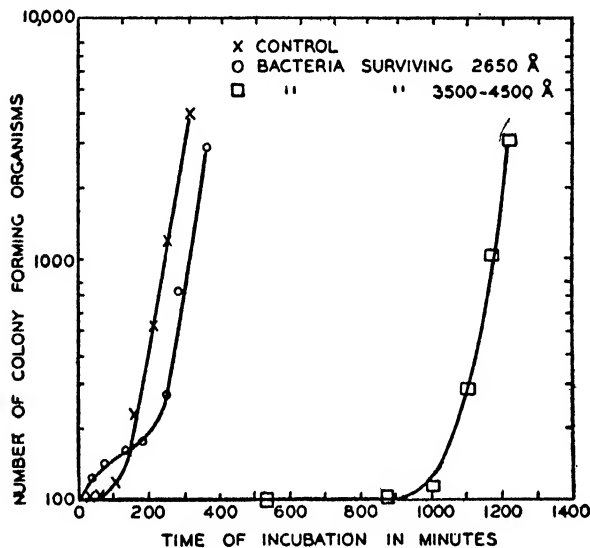


Fig. 9. Growth curves of bacteria. (1) Not irradiated. (2) Bacteria which survived irradiation with 2650 A (S.R. 8%). (3) Bacteria which survived 3500-4500 A (S.R. 8%). (Data taken from Hollaender and Duggar, 1938; Hollaender, 1943.)

It was also learned lately that certain wave lengths, which by themselves have no recognizable effect, if given in combination with wave lengths that produce some effect will emphasize the latter. We are referring to the work done on the effect of near infrared and X-rays. We had observed about 15 years ago that *E. coli* suspended in an ice-cooled nutrient broth could be made to divide at temperatures below 10° C., if irradiated with near infrared radiation of wave length about 10,000 A (1000 to 20,000). In other words, the bacteria absorb the near infrared energy (about 1.2 electron volts) and hold onto it for a time sufficient to activate their enzyme systems and cause some slow divisions to take place.

Kaufmann, Hollaender, and Gay (14) report that near infrared radiation, given alone, has no recognizable effect on chromosome rearrangements as detected by analysis of the salivary-gland chromosomes of *Drosophila melanogaster*. If the near infrared is given before X-ray treatment, the

frequency of detectable X-ray-induced rearrangements is significantly increased as compared with the X-rayed controls.

Swanson and Hollaender (18) found that pre- as well as post-treatment with near infrared (6000-20,000 A) will double the number of X-ray-induced chromosome rearrangements in Tradescantia. Also, pretreatment of *Aspergillus terreus* spores with near
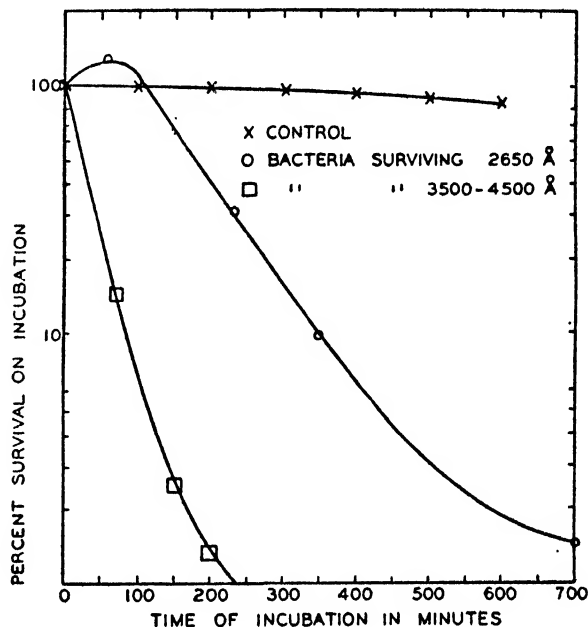


Fig. 10. Survival of bacteria in physiological salt solution. (1) Not irradiated. (2) Bacteria which survived irradiation with 2650 A (8%). (3) Bacteria which survived 3500 to 4500 A (S.R. 8%). (Data taken from Hollaender, 1943.)

infrared will increase significantly the rate of X-ray-induced mutations as compared to that of X-ray controls (12). Near infrared treatment alone will neither produce any recognizable effect on Tradescantia chromosomes nor influence the natural mutation rate of *Aspergillus terreus*.

However, the effects which we recognize when we study individual wave-length ranges may not necessarily be additive if we combine several wave lengths. Much more work is necessary to find out what combinations of wave lengths can do to living cells. Organisms irradiated with sunlight receive not only the biologically effective wave-length range between 2900 and 3150 A, but also the long ultra-violet visible, and a considerable range of the infrared spectrum. The relative proportion of these wave lengths that will affect the organism will vary widely with the time of day, the time of year, the air-mass that the radiation has to penetrate, the amount of dust and smoke in the air, and many other factors. The erythema-producing part will, in general, be more affected by these factors than the other parts of the spectrum.

It is possible that the mutation rate among fungus spores exposed to sunlight is lower than among those exposed to monochromatic 2967 or 3130 A because the presence of the longer lethal wave lengths in sunlight (> 3500 A) does not permit survival of many mutated spores.

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## DISCUSSION

KAUFMANN: Near infrared radiation (wave length ca. 10,000 A) has been found effective in modifying the frequency of X-ray-induced chromosomal rearrangements that are detected by analysis of salivary-gland chromosomes of Drosophila melanogaster. Pretreatment of the spermatozoa "sensitizes" the chromosomes to "breakage" by X-rays; and exposure to near infrared during the period of syngamy and early cleavage also yields a higher frequency of chromosomal rearrangements than from the controls. Analysis of the effect of near infrared radiation when used subsequent to X-rays in the treatment of spermatozoa is complicated by its effect in accelerating those processes that make sperm available for transfer in copulation which were not mature at the time of X-ray treatment. Although it increases the temperature within the organism during the exposure, the effective action of near infrared radiation in facilitating recombination among X-ray-induced breaks is attributed to selective action on certain cellular components and not merely to a temperature effect.

LATARJET: The possibility of direct photochemical action of infrared rays upon components of the genetic fraction of the cell is in agreement with the presence in this fraction of large, relatively fragile molecules. As a matter of fact, in a series of homologous derivatives, the potentials of excitation become generally smaller when substitutions increase the weight of the molecule. For example, in the series of aromatic hydrocarbons, from benzene to graphite, one observes a continuous shift of the absorption and fluorescence spectra towards the long wave lengths when the number of rings increases. In large protein or nucleoprotein molecules, photochemical changes could then be induced by near infrared, as used by Dr. Kaufmann, or as present in sunlight, and possibly even by the long infrared of the isothermic radiation of the body.

LURIA: In connection with the effects of ultraviolet light on the early phases of bacterial growth, I wish to mention a remarkable discovery by Mrs. Witkin. She found that bacteria from a fully grown culture, after irradiation with low doses of ultraviolet light, undergo an immediate process of elongation without cell division, accompanied by increase in the number of nuclear bodies per cell. Giant multinucleated cells are thus formed. The elongation begins long before the nonirradiated control cells divide (lag phase), and leads to the formation in an irradiated culture of much greater amounts of bacterial protoplasm than in the control culture. Each elongated bacterium has the same radiosensitivity as one normal cell.
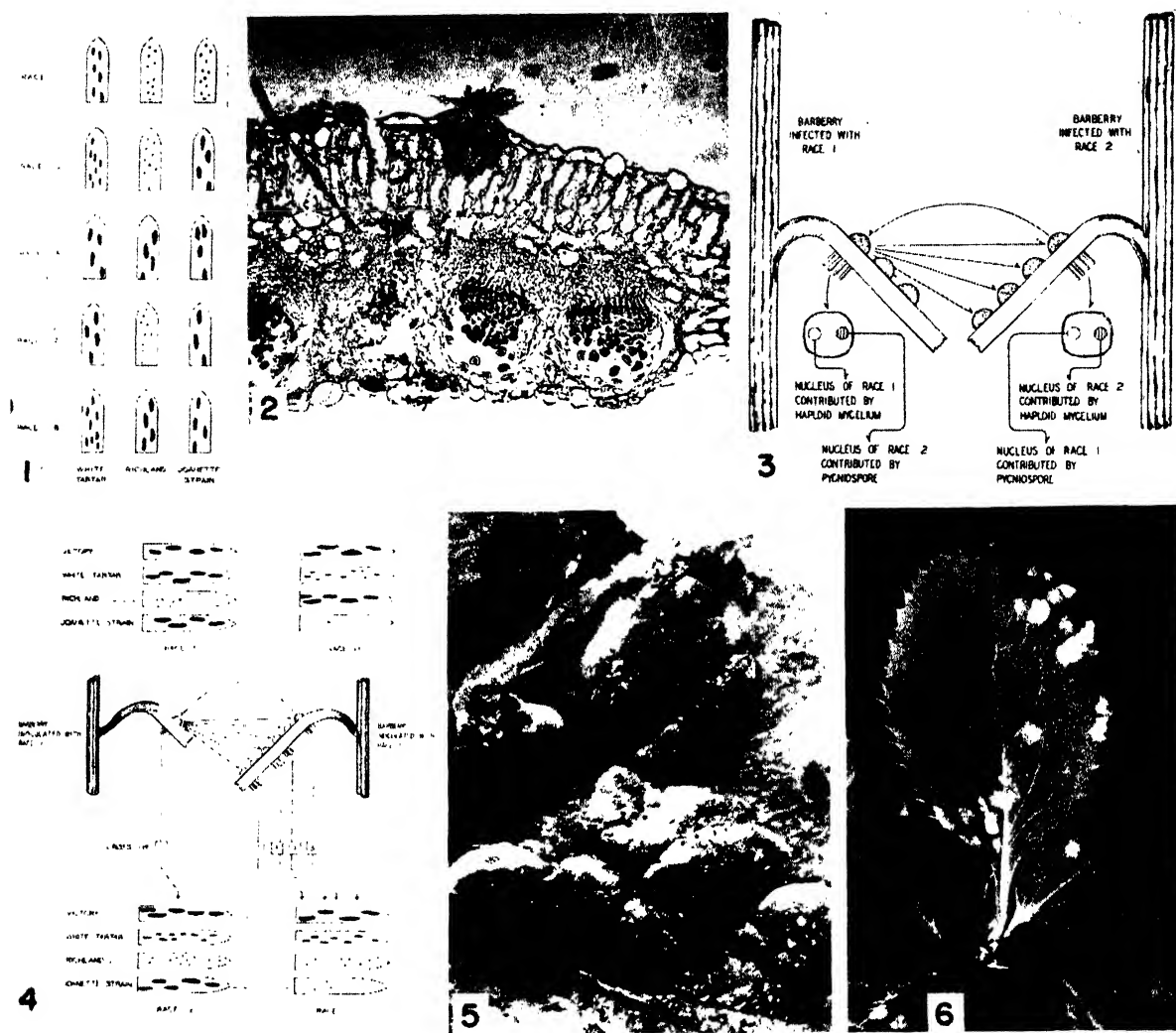
FIG. 1. Diagrammatic representation of the infection types produced by five physiologic races of *P. graminis* var. *Avenae* on the seedling leaves of three oat varieties. The small dots represent sori of type 1, the large ones type 4, and those of intermediate size type 2.

FIG. 2. Photomicrograph of a cross section of a barberry leaf, showing a pycnium and immature aecia.

FIG. 3. Diagram illustrating the "crossing" of two physiologic races. The arrows show the direction of transfer of the pycniospore-containing exudate.

FIG. 4. A diagrammatic representation of five actual crosses between races 7 and 11 of *P. graminis* var. *Avenae*, showing the infection types of the parent races and of the F₁ hybrid races on the differential varieties. In F₁, the type-2 infection on White Tartar is dominant to type 4; and the type-1 infection on Richland is dominant to type 4. On Joanette Strain, the infection type in F₁ resembles that produced by the maternal parent race (i.e., the race receiving a transfer of pycnial exudate)—a fact attributed to cytoplasmic influences.

FIG. 5. Lower side of barberry leaf, showing infections produced by a strain of race 36 of *P. graminis* var. *Tritici* that had lost the ability to produce aecia but produced instead sori of urediospores and teliospores, shown as dark, powdery masses on the surface of the infections. (× *ca.* 10)

FIG. 6. White and normal haploid pustules on barberry leaves, produced by a culture of race 21 of *P. graminis* var. *Tritici*.

# VARIATION AND THE INHERITANCE OF CERTAIN CHARACTERS IN RUST FUNGI[1]

## T. JOHNSON

The rusts differ from most other fungi in that they are obligate parasites. This fact is of importance to the investigator seeking information on the heritable characteristics of this group of fungi; for, unlike most other fungi, the rusts have not, as yet, been grown on artificial media. All such information must, therefore, be obtained from the rust growing on its natural host or hosts. This imposes a limitation on the student of variation or heredity of these organisms, for he is obviously unable to make use of the advantages provided by the pure-culture methods commonly applied to all fungi that can be grown on artificial media.

In common with most other groups of fungi, the rusts are characterized by an immense amount of morphologic variation, evident particularly in the structure of the teliospore, which, for this reason, forms the chief basis of classification. A special feature of the rusts, but one that also applies to most other obligate parasites, is their high degree of host specificity. An autoecious rust is commonly limited to a few species of closely related host plants. A heteroecious rust may attack, and usually does attack, plants of widely different origin—the monokaryotic mycelium attacking one group of closely related plants and the dikaryotic mycelium attacking another and entirely different group of plants. For example, the monokaryotic mycelium of *Coleosporium Solidaginis* (Schw.) Thüm attacks species of Pinus, whereas the dikaryotic mycelium attacks species of Aster and certain other genera of the tribe Astereae. In each of these two stages, however, the rust is highly specific and the host range is relatively narrow. When it is considered that there are in existence 3500 or more species of rusts and that each one has its own particular host or combination of hosts, it is possible to form some idea of the immense range of physiologic variation of the rusts as a group.

Even this does not represent the ultimate in physiologic specificity. In 1894, Eriksson (*13*) discovered that the rust *Puccinia graminis* Pers. could be divided into at least six pathogenically and therefore physiologically distinct classes, which he designated as *formae speciales:* (1) Secalis (on rye, barley, and certain grasses); (2) Avenae (on oats and certain grasses); (3) Tritici (on wheat); (4) Airae (on *Aira caespitosa*); (5) Agrostidis (on Agrostis spp.); (6) Poae (on poa spp.). These *formae*

*speciales* will be herein referred to as varieties of the species *Puccinia graminis.*

In 1916, Stakman and his co-workers (*51, 52*) showed that specialization was carried even farther than this. They found that Eriksson's *forma specialis* or variety Tritici was not a physiologic unit but could be subdivided through the use of appropriate species and varieties of the genus Triticum into many pathogenically distinct strains, of which approximately 200 are known at the present time. Fig. 1 gives a diagrammatic representation of several physiologic races of variety Avenae.

The presence of such physiologically distinct strains, or physiologic races as they are now called, is by no means limited to *P. graminis;* the occurrence of physiologic races is the rule rather than the exception in the rusts that have been investigated.

There can be no question but that the physiologic variability that leads to the development of numerous physiologic races in a rust is a factor favoring the survival of the rust. This has become clearly evident in recent years when man has assumed an active part in guiding plant evolution by the breeding of rust-resistant varieties of economically important plants such as cereals. The aim and objective of such breeding work has been to create plant varieties resistant to all the physiologic races of the rust in question that are present in the region where these varieties are to be grown. Many such efforts have been attended with notable success, and frequently the varieties produced have shown the desired rust resistance—at least for a number of years. But examples have not been wanting of the appearance in a rust of previously unknown physiologic races capable of attacking plant varieties that had been relatively free from attack over a period of years. One such example is the origin and rapid increase in prevalence of race 56 of *P. graminis* var. *Tritici* which, a decade ago, had the effect of converting the moderately resistant wheat variety Ceres into a susceptible variety (*22, 47*). Another is the appearance in Australia, in 1941, of a biotype of race 34 of var. Tritici that has overcome the high resistance formerly shown by the wheat variety Eureka (*32*). A similar adaptation of a rust to resistant host plants appears to have been taking place in North America in the last two or three years in leaf rust of wheat, *Puccinia triticina* Erikss., through the production of new strains capable of attacking wheat varieties that had previously proved resistant to this rust. It has been evident for some time that the physiologic race, as determined by

specially selected groups of differential hosts, is not necessarily the ultimate unit of specialization—for some of the new strains of rust referred to above are substrains or biotypes of previously known physiologic races.

It is the chief purpose of the present paper to explain, as far as present knowledge goes, the mode of inheritance of some of the pathogenic characteristics of physiologic races. Before this can be undertaken, however, it is necessary to review briefly the life cycle of a rust, with special emphasis on the nuclear phenomena. This will be done with particular reference to the species *P. graminis*, which furnishes much of the present knowledge on the inheritance of pathogenic characters.

### THE LIFE CYCLE OF A RUST

The life cycle of *P. graminis* may be regarded as fairly typical of a heteroecious rust. The striking and characteristic feature of such a life cycle is the alternation between a dikaryotic mycelium parasitic (in *P. graminis*) on certain of the Gramineae and a monokaryotic mycelium parasitic on species of Berberis. The life cycle of an autoecious rust does not differ from this essentially except that the two types of mycelium parasitize the same host.

In *P. graminis*, the dikaryotic aeciospores formed on the barberry infect graminaceous hosts. The mycelium thus established is dikaryotic, the paired or "conjugate" nuclei, as they were named by Poirault and Raciborski (45), dividing simultaneously. On this mycelium are formed periodically during the summer the binucleate urediospores which propogate the rust asexually during the summer months. The teliospore formed in late summer is at first binucleate, but the paired nuclei fuse in the mature spore. In the germinating teliospore this nucleus divides and the two daughter nuclei thus produced divide again so that four nuclei are present in the promycelium of the teliospore. These two divisions apparently constitute meiosis, which again initiates the haploid or monokaryotic stage of the rust. The four nuclei are extruded through the delicate wall of the promycelium to take their place in the uninucleate sporidia. Each sporidium, given the opportunity, will produce an infection on the barberry, the mycelium in such an infection being uninucleate. On this mycelium, differentiated structures are soon developed—pycnia (spermogonia) usually at the upper surface of the leaf and aecial primordia (proto-aecidia) near the lower surface (Fig. 2). Uninucleate pycniospores (spermatia), long thought to be degenerate or nonfunctional male gametes, are produced in and exuded from the pycnia. It was shown by Craigie (7, 8, 9, 10) that the pycniospores function as gametes insofar as they are instrumental in re-initiating the binucleate condition essential for the reproduction of the rust. Two deductions may be made from Craigie's experiments. First, that the pycniospores

are the bearers of one of each pair of the nuclei that become associated in the aecial primordium. Second, that the pycnia may be classified into two different intersterility groups, which he designated as (+) and (—). Each haploid thallus (pycnium and aecial primordium) is self-sterile. There is intersterility between any two haploid thalli of the same sign (i.e., any two (+) thalli, or any two (—) thalli). But there is cross-fertility between (+) thalli and (—) thalli.

This situation is closely paralleled by that obtaining in many ascomycetes, as for example, *Sclerotinia Gladioli* Drayton and *Neurospora crassa* Shear & Dodge. In the latter, the spermatiophores are analogous to the pycnia and the bulbils correspond to the aecial primordia, and both these organs are produced on either (+) or (—) mycelia. In *P. graminis*, sex is conditioned by a twofold mechanism similar to that postulated by Lindegren (30) for Neurospora: (1) sex organs, the pycnia (male) and the aecial primordia (female); (2) sterility factors that ensure cross fertilization between thalli.

The discovery of the function of the pycnia provided the investigator with a means of bringing about the association of the nuclei of one physiologic race with those of another; that is, it made possible the "crossing" of physiologic races.

Although the life cycle sketched above is fundamental to the rusts there are many deviations from it, particularly in short-cycled rusts. In *Endophyllum Euphorbiae-sylvaticae* Wint., in which pycnia are present but in which the aeciospore functions as a teliospore, there is evidence that the paired nuclei do not fuse (46). A similar condition has been shown to prevail in *Kunkelia nitens* (Schw.) Arth. (11, 12), in which the aeciospore also functions as a teliospore. The effect on the nuclear phenomena of the absence of pycnia in a rust is a matter of considerable interest. Since pycnia may be regarded as a device to reinstitute the binucleate condition, it might be expected that their absence would be associated with a completely uninucleate life cycle. Such is evidently not the case, though it has been shown, as in *Kunkelia nitens* and in *Uromyces Rudbeckiae* A. & H. (42), that uninucleate strains do occur in some rusts in which pycnia are lacking. Other short-cycled rusts lacking pycnia have apparently developed alternative means of achieving the binucleate condition. In *Puccinia malvacearum* Bert., which is homothallic, the early vegetative mycelium arising from the sporidium is at first uninucleate, but nuclear association occurs some time before the formation of teliospores (2). *Puccinia Arenariae* (Schum.) Wint. (31) appears to be an example of a rust in which the sporidium and the mycelium arising from it are binucleate, the rust therefore being dikaryotic with the exception of the teliospore in which the two nuclei fuse.

The apparent rarity of uninucleate strains in rusts, and the fact that even when pycnia are

absent the rust generally manages to carry on nuclear fusion and meiosis, may be interpreted as evidence of the paramount importance of the sexual process to survival in these organisms.

## Rust Characters Suited to Inheritance Studies

Before it is possible to study inheritance in any organism it is essential that the organism should possess some properties that may be regarded as relatively fixed characters. Variability in the organism is a prime requisite. In rusts, as in most other organisms, crosses must be carried out between different variants of the same species, as distinct species do not intercross readily. Hence it is necessary to consider what variables are present in a species. Suitable morphologic characters are rarely available, because a species is so nearly a morphologic unit. Occasionally, however, there may be present a morphologic character that could serve as the basis of an inheritance study—as, for example, spore size in *P. graminis,* in which the variety Poae has significantly smaller and rounder aeciospores and urediospores and shorter teliospores than the varieties that inhabit cereals (*48*).

Nevertheless, the characters amenable to inheritance studies are for the most part pathogenic. The pathogenic characters encountered in physiologic races are visible expressions of physiologic differences and are characteristic for each physiologic race. Thus, race 36 of the Tritici variety of *P. graminis* produces on the leaves of Mindum wheat a minute rust sorus about the size of a pinhead, surrounded by a halo of necrotic tissue (type 1); whereas race 9 produces on the same wheat a large elongated sorus that may attain the length of half a centimeter or more and is not surrounded by necrosis (type 4). By selfing these races it is possible to discover whether or not they are homozygous for the respective characters. If they prove homozygous—that is, if all the progeny of the selfing reproduces the character in question without appreciable variation—it is possible to cross the two races with good prospects of learning whether one of these infection types is dominant over the other or whether the $F_1$ progeny is characterized by some entirely different type of infection. In such a cross, contrasted pathogenic differences expressed on other hosts may be studied in the same way, until all the pathogenic differences shown by the two races have been subjected to study.

Having determined the characteristics of the $F_1$ progeny of a cross between two races, one may continue the study by selfing the $F_1$ culture. In $F_2$ and subsequent generations, the distribution of the various contrasted pathogenic characters may be determined.

Occasionally the investigator is, by chance, provided with characters rarely, if ever, found in nature. In this category may be included the color abnormalities, to be discussed later, which were among the first rust characteristics to be submitted to genetical study.

## Methods of Crossing and Selfing

As stated earlier, a "cross" between two strains of rust is primarily the bringing into association of a nucleus of one strain with a nucleus of another strain (Fig. 3). A cross may involve more than that, because it is possible that there is also a union of the cytoplasm of the two strains; but there is no definite proof that the nucleus contributed by the pycniospore brings with it any appreciable amount of cytoplasm. In any event, the cross has the effect of bringing the nucleus into a new cytoplasmic environment, which may modify its phenotypic expression. The new strain of rust is during its parasitic existence a dikaryon, and is commonly heterokaryotic. It is with the rust in its dikaryotic phase that most studies on pathogenic behavior and its inheritance are concerned. In crossing two strains of a rust, the pycniospore-containing exudate from a pycnial pustule (presumed to be of monosporidial origin) of strain A is applied successively to several other similar pustules of strain B. The resulting aeciospores contain one nucleus of strain A contributed by the pycniospore and one of strain B contributed by the uninucleate mycelium at the base of the pycnial pustule. The "selfing" of a strain of rust involves merely the intermixing of the pycnial exudate of pustules of the same strain to bring about the various possible nuclear combinations.

Because much of the present knowledge on inheritance of rust characteristics is due to studies on the economically important species *P. graminis,* it is inevitable that full consideration should be devoted to these studies. As stated previously, this species is composed of several physiologically distinct subspecies or varieties. Each of the three varieties that are pathogenic to cereals (the varieties Tritici, Avenae, and Secalis) comprises, in turn, a number of physiologic races. Two kinds of crosses are therefore possible in *P. graminis:* (1) crosses between the different physiologic races within a variety (intravarietal crosses); and (2) crosses between physiologic races of one variety and those of another variety (intervarietal crosses).

## The Selfing of Physiologic Races

Before a discussion of such crosses is undertaken it is desirable to consider briefly the selfing of individual physiologic races. Studies on the selfing of eight physiologic races of the Tritici variety of *P. graminis* (*40*) showed that only one of these races was homozygous for all of the pathogenic properties expressed on the twelve differential hosts used for this rust. The conclusion that most physiologic races of the rust are heterozygous has been confirmed by later studies (*20*). A physiologic race, however, may be heterozygous for pathogenic characteristics ex-

pressed on certain hosts but homozygous for those on other hosts.

Selfing studies soon indicated the dominance or recessiveness of certain infection types. For example, races that rust Kanred wheat heavily always produce progeny of the same type, whereas races that do not rust Kanred (infection type O) fall into two groups (20). Some of the latter produce, when selfed, only races that do not rust Kanred—i.e., they are homozygous for that character. Others produce races of this type and also races that rust Kanred heavily (infection type 4). These facts indicate that inability to rust this wheat (type O) is dominant to the ability to rust it heavily (type 4). It follows that races phenotypically alike are not necessarily alike genotypically; or, in other words, two different cultures of the same race do not necessarily produce identical progeny. An example of this situation is the occurrence, in the progeny of a cross between races 9 and 52, of two genotypically different cultures of race 15. One of these was homozygous, producing only race 15 when selfed; the other was heterozygous and produced a progeny consisting of races 15 and 52 (25).

It is likely that a condition of heterozygosity is also common in rusts other than *P. graminis*. In *Puccinia anomala* Rostr., three physiologic races that were investigated (43) proved to be heterozygous, one of these, when selfed, giving rise to six distinct races. In *P. coronata* Cda. var. *Avenae,* it was shown that race 2 of this rust was heterozygous, as six physiologic races were isolated from a selfing of it (33). Nevertheless, homozygous strains undoubtedly occur in other rusts, as is indicated by the presence in Australia of an evidently homozygous physiologic race of flax rust, *Melampsora Lini* (Pers.) Lév. (54).

## THE CROSSING OF PHYSIOLOGIC RACES
### The Inheritance of Pathogenic Characters

To obtain genetical information from crosses between physiologic races, it is necessary that the pathogenic characters of the strains to be mated show a well-defined contrast; and to simplify the interpretation of progeny studies, it is desirable that the strains be homozygous for these characters.

A cross between races 9 and 36 of *P. graminis*

INFECTION TYPES ON HOST VARIETIES

|  | Kan-red | Ar-nautka | Min-dum | Spel-mars | Ver-nal |
|---|---|---|---|---|---|
| Parents { Race 9 | 0 | 4 | 4 | 4 | 4 |
| Race 36 | 4 | 1 | 1 | 1 | 0 |
| $F_1$ hybrid—Race 17 | 0 | 4 | 4 | 4 | 0 |

var. *Tritici* provides an example of the results of crosses between different races of the same rust variety (20, 41). Above are shown the contrasted characters (infection types) by which the two

parent races differ and also those of the $F_1$ hybrid, race 17.

Several crosses between these two races have produced identical results, the $F_1$ progeny in each case being identified as race 17. Of the pair of contrasted characters on the variety Kanred the type-O infection of race 9 (absence of sori) was dominant to the type-4 infection of race 36 (large sori). The hybrid therefore resembled the race-9 parent in its inability to rust Kanred. On the varieties Arnautka, Mindum, and Spelmars, the hybrid again resembled the race-9 parent in producing large sori on these varieties, the large sori (type 4) therefore being dominant to the small sori (type 1). On the variety Vernal the hybrid resembled the race-36 parent in its inability to rust this variety, the O type produced by race 36 therefore being dominant to the 4 type produced by race 9. Later work (20) has shown dominance and recessiveness of the same characters in crosses between other races, a fact which indicates that these results are not peculiar to crosses between these two races. Selfing studies with the $F_1$ hybrid rust (race 17) showed that the O type on Kanred, which was dominant in $F_1$, reappeared in $F_2$ about three times as often as the recessive 4 type, thus indicating the operation of a single pair of factors. Similarly on the durum varieties Arnautka, Mindum, and Spelmars the dominant 4 type occurred in $F_2$ about three times as frequently as the recessive 1 type, again indicating a pair of alleles. On the variety Vernal, however, the dominant character, type O, appeared about 18 times as frequently in $F_2$ as the contrasted recessive character, type 4, a distribution which suggests that two pairs of factors are operative.

On the assumption that the pathogenicity of the rust was governed by one pair of alleles for Kanred, another pair for the varieties Arnautka, Mindum, and Spelmars, and two pairs of alleles for Vernal, it was expected that the $F_2$ generation would be composed of eight phenotypes; namely, races 1, 9, 11, 15, 17, 36, 52, and 57. All of these races did occur in the $F_2$ generation, and accounted for 307 of the 325 cultures studied, or about 95% of the $F_2$ cultures. Four additional races appeared, however; namely, races 29, 32, 85, and 110. As each of these four races bore a close similarity to some race in the first-mentioned group, they were considered variants of the races they resembled. The actual and theoretical distributions of races in $F_2$ are shown below. The fit is statistically satisfactory.

| Race | 17 | 11 | 1 | 36 | 9 | 57 | 15 | 52 |
|---|---|---|---|---|---|---|---|---|
| Distribution Theor. | 171.4 | 57.1 | 57.1 | 19.0 | 11.4 | 3.8 | 3.8 | 1.3 |
| Actual | 165 | 64 | 47 | 31 | 11 | 4 | 2 | 1 |

The 165 cultures listed for race 17 include 6 of the related race 29; the number listed for race 11

includes 5 cultures of the related race 32; the number for race 9 includes 6 of the related race 85; and the number for race 15 includes 1 of the related race 110.

It may be concluded from this study that Mendelian laws operate in the inheritance of pathogenic characters and, furthermore, that an understanding of the way these characters are inherited may lead to a certain amount of prediction of the physiologic races that may be expected to arise from crosses between known races.

It is probable that the inheritance of pathogenic characters in other rusts follows a somewhat similar pattern. Crosses between races of the Avenae variety of *P. graminis* have shown dominance and recessiveness of certain characters (21), and selfing studies have shown that factors governing these characters segregate and recombine in various ways. A study of a cross between races 22 and 24 of flax rust (*Melampsora Lini*) has shown (14) that on most host varieties the ability to produce a virulent type of infection is inherited as a recessive character. On some varieties, pathogenicity was conditioned by one factor pair; on others, two pairs of factors were operative. In this cross, the presence of many distinct pairs of alleles governing rust behavior led to the production of numerous physiologic races in $F_2$. In a study of 133 $F_2$ cultures, no less than 68 physiologic races were isolated, of which 66 were not previously known.

### Cytoplasmic Inheritance

Although the inheritance of most pathogenic characteristics appears to be governed by Mendelian factors, exceptions to this rule have occurred in interracial crosses in both the Tritici and the Avenae varieties of *P. graminis*. The characteristic feature of these exceptional crosses is that the progenies arising from reciprocal crosses are not identical pathogenically. A typical example is provided by crosses between races 7 and 11 of the Avenae variety of *P. graminis*. Race 7 produces on the oat variety Sevnothree a type-4 infection (large sorus) whereas race 11 produces a type-1 infection (small sorus). All progeny arising from the race-7 side of a cross resembles race 7 by the production of large sori on Sevnothree; and all progeny arising from the reciprocal or race-11 side of the cross resembles race 11 by producing small sori on Sevnothree (21) (Fig. 4). In all other respects the progenies arising from the two sides of the cross are alike. As the nuclear constitutions of the reciprocal hybrids are identical, the factors responsible for the pathogenic difference cannot be nuclear. There is, however, reason to believe that the cytoplasm of the reciprocal hybrids may not be identical. It has never been demonstrated exactly how the pycniospore nucleus reaches the aecial primordium; but in any event it must travel a considerable distance to do so. It may well be that it reaches its destination unaccompanied by the cytoplasm of the pycniospore, which in any case is very scanty owing to the minute size of the spore. If this supposition is correct, each hybrid aeciospore receives from the pycniospore only a nucleus, but from the mycelium of the receptive thallus a nucleus plus cytoplasm. One race used in the cross therefore contributes a nucleus only, while the other contributes a nucleus and cytoplasm. Lamb (27) claimed to have found a confirmation of this view in his cytological studies of the origin of the binucleate condition in *Puccinia Phragmitis* (Schum.) Körn.

In the Tritici variety of *P. graminis* also, where pathogenic differences are shown by some reciprocal hybrids, it has been demonstrated (25, 35) by selfing studies that the pathogenic differences displayed in $F_1$ persist in $F_2$ and $F_3$—all the progeny in these generations showing the cytoplasmic influence. Because of the persistence of this influence, the physiologic races arising from the two sides of a cross are always different, the characters distinguishing the $F_1$ hybrids also distinguishing the $F_2$ and $F_3$ progeny. The absence of any clear-cut segregation for characters affected by the cytoplasm would suggest that, as far as these characters are concerned, nuclear factors are overbalanced by the influence of the cytoplasm. In the same crosses, characters not affected in $F_1$ by the cytoplasm show definite Mendelian segregation in $F_2$ and later generations.

### The Inheritance of Urediospore Color in P. graminis

It may seem anomalous to discuss the inheritance of urediospore color in *P. graminis*, an organism in which urediospore samples collected in nature usually show no appreciable differences in color. The opportunity of studying the inheritance of urediospore color was provided by the production in the variety Tritici, presumably through mutation, of two strains of aberrant color (34). One of these, a strain with orange urediospores, arose by mutation from a normal red-spored culture of race 9. The other, a strain with grayish-brown spores, was isolated from cultures derived from aeciospores. Both mutations represented a loss of pigment. In the orange strain there was an absence of pigment from the spore wall, the carotinoid pigment in the spore therefore being clearly visible and accounting for the orange color of the spores. In the grayish-brown strain there was a virtual absence of the carotinoid pigment from the cytoplasm.

In all crosses between these two strains, the urediospores of the $F_1$ progeny were of the normal red color. A selfing of the $F_1$ progeny resulted in production in $F_2$ of spores of four color classes: red, orange, grayish-brown, and white. In the white spores, spore-wall pigment and cytoplasmic pigment were both absent. The size of the color classes suggested a 9:3:3:1 ratio. The inheritance of urediospore color could accordingly be explained by

assuming the presence of two pairs of color factors, GG governing pigmentation of the spore wall and YY governing that of the cytoplasm, both present in spores of normal, red color. The constitution of the orange spores would then be ggYY and that of the grayish-brown GGyy. The white spores would represent the double recessive ggyy.

This two-factor scheme is perhaps not applicable to all color inheritance in *P. graminis*. Minute variation in the color of stem rust collected in nature (*15, 53*) and the occasional occurrence in other crosses or selfing studies of different shades of orange or grayish-brown color suggest the existence of other genes that influence urediospore color.

### CROSSES BETWEEN VARIETIES OF PUCCINIA GRAMINIS

The inheritance studies thus far dealt with are concerned with crosses between physiologic races of the same variety (i.e., intravarietal crosses). In such crosses interfertility betweeen different races is high and crosses are readily obtained. In crosses between varieties (e.g., Tritici × Avenae, Tritici × Agrostidis) interfertility is often low, the degree of sterility differing according to which varieties are crossed. By making crosses between the varieties Tritici and Secalis and the varieties Tritici and Agrostidis, Stakman *et al.* (*49*) first showed that intervarietal crosses are possible. Subsequent crosses by Levine *et al.* (*28, 29*) and by Johnson *et al.* (*18, 24*) and unpublished work by the latter investigators have thrown some further light on intervarietal hybridization.

It is now evident that the degree of fertility is not the same between all varieties. The variety Tritici is more highly interfertile with the Secalis variety than with varieties Avenae, Agrostidis, and Poae. This is not surprising in view of the fact that varieties Tritici and Secalis have in *Hordeum vulgare* L. a common host, a fact indicating a rather close pathogenic relationship. Crosses have likewise indicated that varieties Agrostidis and Poae, which have common hosts in some species of Poa, are rather readily interfertile.

Regarding the pathogenic properties of $F_1$ hybrids of intervarietal crosses, it is possible to arrive at certain generalizations. Most commonly, a hybrid between two varieties possesses a wider host range than either parent variety, but this widening of the host range is accomplished at the expense of pathogenic vigor. Thus Avenae × Tritici hybrids can attack both wheat and oats; but they attack wheat less vigorously than does the variety Tritici and oats less vigorously than does the variety Avenae. Secalis × Tritici hybrids generally attack wheat and rye lightly but barley vigorously; one such hybrid, however, was found to attack all three hosts rather severely (*28*).

From the intervarietal crosses thus far made it

may be inferred that there are two causes that tend to maintain intact the identity and individuality of the existing varieties of *P. graminis*. One is the considerable intersterility between varieties. The other is the lack of pathogenic vigor in intervarietal hybrid rusts, which rarely find a host to which they are particularly adapted.

### INBREEDING AND MUTATION

Most rusts, as collected in nature, show a remarkable uniformity in appearance. Abnormalities in color or other visible characters are rare. When, however, they are taken out of their natural environment and subjected to study under laboratory conditions it soon becomes evident that much deviation from the normal can take place.

In *P. graminis*, strains of atypical color are rarely found in nature. Nevertheless such strains, particularly those of a grayish-brown urediospore color, are not very uncommon in cultures established from aeciospores. In nature such strains also undoubtedly arise from aeciospores but are evidently less fitted for survival than those of normal color.

The inbreeding of physiologic races—i.e., the repeated selfing generation after generation of the same strain—has frequently led to the appearance of unsuspected abnormalities. These are generally of a kind that would be detrimental to the survival of the rust (*19*). In the progeny of the cross, race 36 × race 9 of the variety Tritici, the following abnormalities were noted, chiefly in the $F_3$ generation: decrease in vigor of sporulation, expressed in partial or complete loss of ability on the part of the uredia to break out through the epidermis; failure of some strains to produce uredia at high temperatures; loss of ability to produce aecia; production of uredia and telia on the barberry by some strains that had lost the ability to form aecia (Fig. 5). Some of the urediospores produced by these strains were uninucleate (*37*).

The production of teliospores on the barberry is interesting from the evolutionary point of view. It lends support to the idea advocated by Jackson (*17*) that microcyclic forms arise from the haploid generation of heteroecious eu-forms through the replacement of aecia by telia. Short-cycled species thought to have originated in this manner are known as "correlated species." The production of telia on the barberry may perhaps be interpreted as evidence of a tendency on the part of *P. graminis* towards the formation of such a species.

Abnormal characteristics are by no means limited to the dikaryotic phase of a rust. Cotter (*5*) observed on barberry infected with the Agrostidis variety of *P. graminis* the production of white pycnia with colorless exudate, the intermixing of which led to the formation of white aecia. Application to white pycnia of the exudate of normal yellow pycnia resulted in the formation of normal yellow aecia, as did also the reciprocal transfer of exudate from

white to yellow pycnia. Evidently, yellow—that is, normal—aecial color is dominant to white.

In the selfing of race 21 of *P. graminis* var. *Tritici* two types of haploid pustules were produced in about equal numbers in barberry (*23, 36*) (Fig. 6). Pustules of one type were normal in appearance and produced aecia following the intermixing of their exudate. Pustules of the other type were almost white and produced only a few scattered pycnia or none at all. Aecia were rarely formed in the white pustules and were usually limited to rudimentary structures, but uredia and telia were formed in some of these pustules after a prolonged period. When exudate from the white pustules was used to dikaryotize normal haploid pustules of other races, the resulting progeny, when selfed, gave rise to white and normal haploid pustules on barberry in about equal numbers. Normal × normal matings produced rust that gave rise to normal haploid pustules only, but white × white matings were sterile. Evidently a mutation had taken place in the original culture of race 21 that affected one of the two conjugate nuclei. This mutation most probably occurred in the uredial stage. During meiotic divisions in the germinating teliospores, the mutant factor segregated so that half the sporidia gave rise to white and half to normal pustules.

The abnormal characteristics brought to light by the inbreeding of apparently normal strains of rust may in most cases be attributed to mutations that had taken place at some time in the past. These remained unexpressed because of their recessive nature until the mutant factors were brought together in a homozygous condition by inbreeding. Abnormal strains thus encountered in the laboratory are rare in nature, partly because inbreeding probably occurs rarely under natural conditions and partly because such strains are not well adapted for survival. Besides, the production of abnormal strains of rust is not an inevitable consequence of inbreeding, as many inbred strains have shown no visible evidence of abnormality.

The part played by mutation in the evolutionary changes taking place in a rust is difficult to assess. It is less easy to demonstrate a mutation in rusts than in fungi that can be grown on artificial media. Because rusts must be grown on their natural hosts in greenhouses, possibilities of admixture and contamination are usually present; and it is therefore difficult to prove that a suspected mutant may not have been a mere admixture of some other strain.

Nevertheless, indisputable mutants have been found occasionally, both under natural conditions and in the laboratory. Among mutants found in nature may be mentioned a white-spored uredial strain of *Melampsora Euphorbiae* Schub. var. *Pepli* found by Pieschel (*44*) in Germany in 1931 and again encountered in the same locality three years later. Color mutants collected in nature have also been reported by Johnston (*26*) for *P. triticina,* by

Garbowski (*15*) for *P. graminis* var. *Tritici,* and by Newton and Johnson (*39*) for *P. graminis* var. *Avenae.* A mutant reported by Brown (*4*) in *Puccinia Helianthi* Schw. displayed whitish haploid infections and grayish urediospores, showed differences in spore viability and pathogenicity, and was, furthermore, virtually intersterile with the strain that gave rise to it.

Among mutations occurring in rust under study in the laboratory may be mentioned the mutation from red to orange urediospore color reported by Newton and Johnson (*34*) to have occurred in race 9 of *P. graminis* var. *Tritici,* and a similar color mutation in race 34 of the same rust reported by Waterhouse (*53*). Similar color mutations have been reported by Cotter and Levine (*6*) for the Secalis variety of this rust and by D'Oliveira (*43*) for *P. anomala,* the last mentioned being also accompanied by a change in pathogenicity. All these mutations involve a loss of pigment in the urediospores; it is therefore apparent that this type of mutation is not uncommon in the rusts.

Many mutations affecting pathogenicity have been recorded, particularly in the cereal rusts. In *P. graminis* var. *Tritici* Stakman *et al.* observed four separate mutations in the same physiologic race (*49*). Newton and Johnson reported two separate instances, one in var. Tritici (*38*) and another in var. Avenae (*39*), of a physiologic race being replaced by another race apparently through a recurrence of the same mutation. A striking example of repeated recurrence of the same mutation was recorded by Gassner and Straib (*16*) for *P. glumarum* (Schm.) Erikss. & Henn. In their study, there occurred no less than 34 recurrent mutations resulting in identical mutants more highly virulent towards certain hosts than was the original race from which they arose. The fact that several instances are on record of the recurrence of the same mutation would suggest that mutation in the rusts not infrequently takes this form.

Mutation is probably more common in the rusts than is generally suspected, but much less common than in many other fungi. In this respect, the dikaryotic nature of the rusts lends to these organisms a stability somewhat comparable to that conferred on higher plants by the diploid nucleus.

Mutant factors in the rusts are probably for the most part recessive. In the dikaryotic phase of a rust a point mutation affecting a homozygous condition would have to occur twice, once for each locus, before it affected the character in question. Such mutations are probably rare; but it is possible that through some abnormal process in mitosis —for example, nondisjunction—the two daughter chromosomes arising from the chromosome bearing the mutant gene might pass to the same pole, thus giving rise to a cell homozygous for the mutant factor. A mutation, however, even if it is not immediately expressed may have potential importance;

for it may reach expression at any time in a new rust generation developing from the infected alternate host. It has indeed been shown by Stakman *et al.* *(50)* that *P. graminis* arising from the infected alternate host, the barberry, shows many times greater variability than is shown by general uredial collections of this rust. It is the function of the sexual phase to give the fullest possible scope to the variability produced by mutation.

It is possible that there exist in the rusts other sources of variation than those that have been discussed. The possibilities of sudden phenotypic changes due to polyploidy or chromosome aberrations are thus far unexplored. Cytologic studies have been greatly handicapped by the extremely minute size of rust chromosomes, the number of which is not even known except perhaps in a few species. It is generally supposed that the close association of the nuclei of the dikaryon precludes the possibility of new nuclear associations occurring during the dikaryotic phase. However, as it has been shown *(3)* that in *P. Helianthi* a dikaryotic mycelium can dikaryotize a monokaryotic one, there is just a possibility that in some rusts nuclear exchanges may also occur between dikaryotic mycelia. It has, indeed, been suggested by Allen *(1)* on cytological grounds that in *P. triticina* there may take place anastomoses of germ tubes or hyphae which would bring together nuclei of different physiologic races. This matter is one that might well be investigated, for if new nuclear associations should take place in the dikaryotic phase much variation would be expected to occur even in the absence of an alternate host.

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## DISCUSSION

Sonneborn: The case of maternal determination of an inherited character reported by Dr. Johnson is at least closely parallel, so far as it goes, to the inheritance of Killer in Paramecium. In Paramecium the cross of a Killer ($KK$ plus kappa) by a sensitive ($KK$) also shows that the Killer character strictly follows the cytoplasm. Only when Killers are crossed to $kk$ sensitives does the genic role become apparent. In this case there is no evidence to indicate an inhibitory action of the cytoplasm upon the action of the genes, and, in view of the seeming parallel to the situation described by Dr. Johnson in the rust, such an assumption would at present seem unwarranted in that material.

# DISTINCTIVE CONSTITUENTS OF TUMOR CELLS AND THEIR POSSIBLE RELATIONS TO THE PHENOMENA OF AUTONOMY, ANAPLASIA, AND CANCER CAUSATION[1]

## JOHN G. KIDD

Several years ago the fact was reported that the cells of the Brown-Pearce rabbit carcinoma—a typical transplantable cancer of unknown cause—when ground in a mortar and extracted with isotonic saline solution will regularly yield a distinctive substance which is identifiable by serological means (53, 54, 55, 76). This material has not been detectable in extracts of other rabbit tissues, either normal or neoplastic; upon analysis it proved to have certain physical and chemical properties remarkably similar to those of the viruses, notably a large particle size and weight as determined by ultrafiltration and ultracentrifugation, and other properties which suggest that it is probably a protein and perhaps a ribose nucleoprotein (54, 76). The subsequent studies of Claude and others have shown, however, that these physical and chemical properties are likewise shared by particulate components of a wide variety of normal tissues (15-20, 48, 37, 38, 102, 111), though it has become plain that the Brown-Pearce tumor cell constituent can be readily distinguished from the particulate components of normal rabbit tissues by serological means (76).

In this Symposium I propose to review briefly the properties of the distinctive constituent of Brown-Pearce carcinoma cells, to give some recently acquired data which indicate that it may play a part in the neoplastic activities of its host cells, to bring out the fact that another distinctive sedimentable material has recently been procured from malignant cells of a different sort which has not been extractable from their benign prototypes, and finally to discuss quite briefly and tentatively certain relations that these findings may have to the larger phenomena of tumor causation.

### Properties of the Distinctive Constituent of the Brown-Pearce Carcinoma Cell

The findings of an actual experiment (Table 1) make plain the fact that sera procured from certain rabbits implanted with the Brown-Pearce carcinoma contain an antibody which will fix complement specifically in mixture with extracts of that growth though it does not react at all with similar extracts of other rabbit neoplasms, with those of normal rabbit tissues (kidney, liver, spleen, red bone mar-

row), or with those of rabbit pus. In many other tests of this kind the specific antibody has regularly reacted in high titer with extracts of the Brown-Pearce carcinoma, and it has in every case failed to react with extracts of a wide variety of other rabbit tissues both normal and neoplastic—some of these having been procured from normal animals, others from hosts in which the Brown-Pearce tumor had grown progressively and metastasized, and still others from animals in which the Brown-Pearce carcinoma had regressed. Furthermore, the antibody has not agglutinated the erythrocytes of rabbits in any of the categories just mentioned (76).

To illustrate further the specificity of the reaction, the fact may be mentioned that extracts of Brown-Pearce carcinoma tissue, brought to a final dilution of 1:320 to 1:1280 with saline, have invariably fixed complement in mixture with sera containing high titers of the specific antibody; while extracts of normal tissues and of other neoplasms have failed to do so in all dilutions of the tissue extracts upwards from 1:10 (the most concentrated amount feasible in the test). Still the point deserves emphasis that the distinctive constituent or some precursor of it could exist in normal tissue cells in comparatively small amounts and thus escape detection by the methods employed.

In previous studies much use was made of a special breed of rabbits—blue-cross hybrids of the Rockefeller Institute strain—to provide the specific antibody. Certain of the blue-cross rabbits developed the antibody in high titer following the implantation of Brown-Pearce carcinoma cells or the injection of cell-free extracts thereof, while other individuals of the same inbred strain failed to do so (76); market-bought animals proved inferior as a source of useful antisera since they often developed, along with the specific Brown-Pearce antibody, other antibodies that react with sedimentable constituents of a wide variety of tissues, both normal and neoplastic (36, 76), about which more will be said later on. The nature of the constitutional factors responsible for this selective production of antibodies has remained undisclosed. Recently, however, a strain of chocolate-Dutch hybrid rabbits has been procured in which the specific Brown-Pearce antibody appeared in high titer in every one of eleven animals following implantation with the Brown-Pearce carcinoma. The implications of the finding are being pursued.

TABLE 1. TESTS WITH SERA PROCURED FROM RABBITS IMPLANTED WITH THE BROWN-PEARCE CARCINOMA IN MIXTURE WITH ANTIGENS MADE FROM VARIOUS RABBIT TISSUES

| Serum from Rabbit No. | Source of antigens (rabbit tissues) | Complement fixation tests Dilution of antigen | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 |
| 4–19 | Normal kidney | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal liver | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal spleen | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal bone marrow (red) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-NaCl | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-BCG | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-aleuronat | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-saponin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Brown-Pearce carcinoma | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | V2 carcinoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | RSI sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | Kato sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| 5–47 | Normal kidney | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal liver | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal spleen | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal bone marrow (red) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-NaCl | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-BCG | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-aleuronat | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-saponin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Brown-Pearce carcinoma | ++++ | ++++ | ++++ | ++++ | ++++ | + |
| | V2 carcinoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | RSI sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | Kato sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| 5–51 | Normal kidney | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal liver | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal spleen | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal bone marrow (red) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-NaCl | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-BCG | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-aleuronat | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-saponin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Brown-Pearce carcinoma | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| | V2 carcinoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | RSI sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | Kato sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| 5–53 | Normal kidney | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal liver | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal spleen | 0 | 0 | 0 | 0 | 0 | 0 |
| | Normal bone marrow (red) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-NaCl | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-BCG | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-aleuronat | 0 | 0 | 0 | 0 | 0 | 0 |
| | Pus-saponin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Brown-Pearce carcinoma | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| | V2 carcinoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | RSI sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |
| | Kato sarcoma | 0 | 0 | 0 | 0 | 0 | 0 |

The sera were all diluted 1:4 with saline; they had been heated at 65° C. for 30 minutes to destroy complement and any natural antibody that may have been present.

The antigens were made from frozen tissues. 0.9% NaCl, aleuronat and starch, and 0.1% saponin, respectively, had been injected intraperitoneally into normal rabbits to elicit pus cells, which had been harvested by washing out the cavities with 3.8% sodium citrate after 24 to 48 hours. BCG pus was procured by repeatedly injecting the organism, generously provided by Dr. Jules Freund, subcutaneously into rabbits.

(Reprinted from *J. Exp. Med.* 82: 41–63. 1945.)

Tables 2 and 3 provide data which show that the distinctive constituent of the Brown-Pearce carcinoma has a large particle size and weight; it is readily sedimentable by high-speed centrifugation and is held back by collodion membranes having pore diameters of 348 mμ and less.

Several findings indicate that the serologically active substance is probably a protein. The results

TABLE 2. COMPLEMENT FIXATION TESTS WITH A CENTRIFUGALIZED EXTRACT OF THE BROWN-PEARCE TUMOR

| Extract of Brown-Pearce tumor* | | | Brown-Pearce antiserum 71 | Normal rabbit serum 1-07 | Controls (no serum) |
|---|---|---|---|---|---|
| Centrifugation | Material used | Dilution | | | |
| (a) 4400 R.P.M., 30 min. | Supernatant | 1:20 | ++++ | 0 | 0 |
| | | 1:40 | ++++ | 0 | 0 |
| | | 1:80 | ++++ | 0 | 0 |
| | | 1:160 | ++++ | 0 | 0 |
| | | 1:320 | +++± | 0 | 0 |
| (b) First high-speed centrifugation (portion of supernatant of (a) centrifuged at 30,000 R.P.M. for 130 min.) | Sediment resuspended | 1:20 | ++++ | 0 | 0 |
| | | 1:40 | ++++ | 0 | 0 |
| | | 1:80 | ++++ | 0 | 0 |
| | | 1:160 | ++++ | 0 | 0 |
| | | 1:320 | ++ | 0 | 0 |
| | Supernatant | 1:20 | 0 | 0 | 0 |
| | | 1:40 | 0 | 0 | 0 |
| | | 1:80 | 0 | 0 | 0 |
| | | 1:160 | 0 | 0 | 0 |
| | | 1:320 | 0 | 0 | 0 |
| (c) Second high-speed centrifugation (portion of the resuspended sediment of (b) centrifuged again at 30,000 R.P.M. for 100 min. after intermediate low-speed centrifugation) | Sediment resuspended | 1:20 | ++++ | 0 | 0 |
| | | 1:40 | ++++ | 0 | 0 |
| | | 1:80 | ++++ | 0 | 0 |
| | | 1:160 | ++++ | 0 | 0 |
| | | 1:320 | + | 0 | 0 |
| | Supernatant | 1:20 | 0 | 0 | 0 |
| | | 1:40 | 0 | 0 | 0 |
| | | 1:80 | 0 | 0 | 0 |
| | | 1:160 | 0 | 0 | 0 |
| | | 1:320 | 0 | 0 | 0 |
| Controls (no antigen) | | | | 0 | 0 |

2 units of complement in all tubes.
Sera diluted 1:4.

* Muscle tumors of D.R. 5-03.
(Reprinted from *J. Exp. Med.* 71: 335–371. 1940.)

of the experiment recorded in Table 4, for example, show that the antigen of the Brown-Pearce carcinoma is inactivated by heating at 65° C. for 30 minutes, this treatment also inactivating the virus-antigen derived from the Shope rabbit papilloma. The inactivation of a highly purified preparation of the distinctive constituent of the Brown-Pearce carcinoma at pH 4.0 and below and pH 10.0 and above are shown in Table 5. From the findings set down in Table 6 it will be seen that trypsin and chymotrypsin will readily inactivate purified preparations of the substance derived from the Brown-Pearce carcinoma cell. In all of these respects the findings have been constant in repeated experiments.

Other data, which will not be given in detail, have shown that alcoholic extracts of the Brown-Pearce carcinoma do not contain detectable amounts of the serologically active material (54). This solvent—and chloroform, acetone, and ether as well—will extract appreciable quantities of lipids from dried preparations of the purified antigen; yet the lipid extracts, suspended in saline in a variety of ways, have shown no ability to react with the specific Brown-Pearce antibody, while the fractions

remaining after extraction with some of the organic solvents proved quite active in this respect when resuspended in saline.

In unreported experiments, Claude and I found that watery extracts of the Brown-Pearce tumor, purified by repeated differential centrifugations and containing the serologically active substance in large amounts, are made up of small spheroid particles in suspension which proved just visible under the dark-field microscope and seemed to be of fairly homogeneous size. About 50% of the mass of these particles was made up of alcohol- and ether-extractable lipids, the nonlipid fraction having about 14% nitrogen, 50% carbon, and 1% phosphorus, and giving strongly positive tests for ribonucleic acid.

The findings just mentioned provide evidence that the distinctive constituent either is associated with, or concurs with, the microsomes of Brown-Pearce carcinoma cells, these elements being identical, so far as the chemical tests indicate, with those isolated from normal tissue cells (14-18). Serological tests with a natural heat-labile antibody that reacts with sedimentable constituents of a wide variety of normal and neoplastic tissue cells corroborate the findings. For the natural antibody

reacts with the sedimentable constituents of Brown-Pearce carcinoma cells even after these have been washed repeatedly in differential centrifugations, as detailed studies have shown (58), though it is noteworthy in this relation that the serologically active component with which the specific Brown-Pearce antibody reacts is far more stable than is the concurrent or associated substance with which the natural antibody reacts: the specific Brown-Pearce constituent remains active after periods of several months when kept at 4° C. and at neutral reaction while the substance with which the natural antibody reacts loses most of its serological reactivity within a few days at 4° C. and pH 7.4. The relationship

TABLE 3. COMPLEMENT FIXATION TESTS WITH AN EXTRACT OF THE BROWN-PEARCE TUMOR FILTERED THROUGH COLLODION MEMBRANES OF VARIOUS PORE DIAMETERS

| Filtration of antigen (A.P.D. of membrane) | Dilution of antigen | Brown-Pearce antiserum 71 | Normal serum 5-00 A | Antigen controls (no serum) |
|---|---|---|---|---|
| Unfiltered | 1:20 | ++++ | 0 | 0 |
| | 1:40 | ++++ | 0 | 0 |
| | 1:80 | ++++ | 0 | 0 |
| | 1:160 | ++++ | 0 | 0 |
| | 1:320 | ++++ | 0 | 0 |
| | 1:640 | +++ | 0 | 0 |
| 571 mμ | 1:20 | ++++ | 0 | 0 |
| | 1:40 | ++++ | 0 | 0 |
| | 1:80 | ++++ | 0 | 0 |
| 397 mμ | 1:20 | ++++ | 0 | 0 |
| | 1:40 | ++++ | 0 | 0 |
| | 1:80 | +++± | 0 | 0 |
| 383 mμ | 1:20 | ++++ | 0 | 0 |
| | 1:40 | ++++ | 0 | 0 |
| | 1:80 | +++± | 0 | 0 |
| 348 mμ | 1:20 | 0 | 0 | 0 |
| | 1:40 | 0 | 0 | 0 |
| | 1:80 | 0 | 0 | 0 |
| 265 mμ | 1:20 | 0 | 0 | 0 |
| | 1:40 | 0 | 0 | 0 |
| | 1:80 | 0 | 0 | 0 |
| 193 mμ | 1:20 | 0 | 0 | 0 |
| | 1:40 | 0 | 0 | 0 |
| | 1:80 | 0 | 0 | 0 |
| 154 mμ | 1:20 | 0 | 0 | 0 |
| | 1:40 | 0 | 0 | 0 |
| | 1:80 | 0 | 0 | 0 |
| 139 mμ | 1:20 | 0 | 0 | 0 |
| | 1:40 | 0 | 0 | 0 |
| | 1:80 | 0 | 0 | 0 |
| Serum controls (no antigen) | | 0 | 0 | |

2 units of complement in all tubes.
Sera diluted 1:8.
Antigen, saline-broth extract of Brown-Pearce tumors from 2 rabbits.
(Reprinted from *J. Exp. Med.* 71: 335–371. 1940.)

between the distinctive constituent and the microsomes of Brown-Pearce carcinoma cells, as pointed out in previous papers (36, 58, 76), has more than casual interest because of the fact that the filtrable agents responsible for fowl sarcomas and related growths are also associated with, or concur with, cellular constituents of similar sort.

TABLE 4. EFFECT OF HEAT ON THE ANTIGENS DERIVED FROM THE BROWN-PEARCE TUMOR AND THE RABBIT PAPILLOMA

| Heating (30 min.) | Dilution | Brown-Pearce tumor antigen (D.R. 7-33) | Papilloma antigen (W.R. 1-30) |
|---|---|---|---|
| °C. Unheated | 1:20 | ++++ | ++++ |
| | 1:40 | ++++ | ++++ |
| | 1:80 | ++++ | ++++ |
| | 1:160 | ++++ | ++ |
| | 1:320 | +± | 0 |
| 56 | 1:20 | ++++ | ++++ |
| | 1:40 | ++++ | ++++ |
| | 1:80 | ++++ | ++++ |
| | 1:160 | +++± | ++ |
| | 1:320 | ± | 0 |
| 60 | 1:20 | ++++ | ++++ |
| | 1:40 | ++++ | ++++ |
| | 1:80 | +++± | ++++ |
| | 1:160 | 0 | ++ |
| | 1:320 | 0 | 0 |
| 65 | 1:20 | 0 | 0 |
| | 1:40 | 0 | 0 |
| | 1:80 | 0 | 0 |
| | 1:160 | 0 | 0 |
| | 1:320 | 0 | 0 |
| 70 | 1:20 | 0 | 0 |
| | 1:40 | 0 | 0 |
| | 1:80 | 0 | 0 |
| | 1:160 | 0 | 0 |
| | 1:320 | 0 | 0 |

2 units of complement in all tubes.
Brown-Pearce antiserum D.R. 5-04, 1:16 mixed with Brown-Pearce antigen.
Papilloma antiserum D.R. F 4, 1:32 mixed with papilloma antigen.
Antigens, 1:20 saline extracts heated as such then diluted as indicated.
(Reprinted from *J. Exp. Med.* 71: 335-371. 1940.)

### Pathogenicity Tests with Extracts of Brown-Pearce Carcinoma Cells

The properties of the distinctive constituent of Brown-Pearce tumor cells—notably its antigenicity, its large particle size and weight, and its lability to heat, to changes in pH, and to the action of proteolytic enzymes, which indicate that it is probably a protein—suggested strongly the possibility that it might be a virus or virus-like substance. Hence a considerable number of experiments were done several years ago to learn whether extracts that contained the serologically active constituent in large amounts would give rise to tumors or other lesions upon injection into rabbits. The extracts

were injected intravenously, and directly into the skin, testicles, and other tissues of normal rabbits, and in later experiments into the skin and other tissues of tarred rabbits and of those carrying ex-

| pH* | Complement Fixation Tests Dilution of Antigen | | | |
|---|---|---|---|---|
| | 1:20 | 1:40 | 1:60 | 1:90 |
| 1.0 | 0 | 0 | 0 | 0 |
| 2.0 | 0 | 0 | 0 | 0 |
| 3.0 | 0 | 0 | 0 | 0 |
| 4.0 | 0 | 0 | 0 | 0 |
| 5.0 | ++++ | +++± | + | 0 |
| 6.0 | ++++ | ++++ | ++++ | ++++ |
| 7.0 | ++++ | ++++ | ++++ | ++++ |
| 8.0 | ++++ | ++++ | ++++ | +++± |
| 9.0 | ++++ | ++++ | ++++ | + |
| 10.0 | ++++ | ++ | 0 | 0 |
| 11.0 | ++++ | ++ | 0 | 0 |
| 12.0 | 0 | 0 | 0 | 0 |
| 13.0 | 0 | 0 | 0 | 0 |

* Aliquots of the suspension of the purified material in neutral water were brought to the indicated pH levels with NaOH and HCl. After standing 1 hour at room temperature the various samples were neutralized, rendered isotonic with added NaCl solution (18%), and tested for ability to fix complement according to a standardized technique (58).

perimentally induced syphilitic chancres. In no case did a lesion develop (54).

While it is plain that the material was non-pathogenic under the circumstances of the experiments, it was pointed out at the time that the tests were far from exhaustive; indeed, when reviewed for the purposes of this Symposium and in the light of what has since been learned about the complexity of factors influencing the activities of cellular constituents, the negative findings seemed wholly inconclusive as precluding the possibility that the serologically active substance may be responsible

for the neoplastic activities of the tumor cells under the conditions of its natural association with them.

### Suppression of Growth of Brown-Pearce Carcinoma Cells by the Antibody That Reacts Specifically with Their Distinctive Constituent

To study further the nature and possible functions of the specific Brown-Pearce substance, indirect methods were employed: tests were made to learn whether the antibody that reacts specifically with the distinctive substance has any effect on the living tumor cells that harbor it. The antibody suppresses their growth, as two types of experiments have shown (57).

From the data of Fig. 1, it will be seen that Brown-Pearce cells incubated for 2 hours in vitro with sera containing the specific antibody in high titer failed to grow, or grew but poorly, upon subsequent implantation into susceptible animals; whereas the tumor cells were unaffected by normal rabbit sera or by those of rabbits carrying cancers of other sorts. In the second type of experiment (Fig. 2), rabbits whose sera contained the specific antibody as result of previous injections of cell-free extracts containing the serologically active material, proved resistant to implantations with the tumor cells, while rabbits that had failed to develop the antibody under such circumstances were notably susceptible. In several experiments of both kinds the findings were the same (57). They provide an indication that the distinctive constituent may play an important part in the proliferative activities of the Brown-Pearce carcinoma cells, and they are not devoid of implications for research in the chemotherapy of cancer, about which more will be said in the Discussion.

The fact deserves mention at this point that the effects of the specific antibody in suppressing the growth of Brown-Pearce tumor cells are wholly distinct from those of the unknown factor or factors that frequently bring about regression of the

| Brown-Pearce antigen 1:10* plus equal parts of | Complement Fixation Tests (Brown-Pearce antiserum 5:47, 1:8) | | | | | |
|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 |
| 1. Dilute phosphate buffer (0.01 M, pH 7.4) | ++++ | ++++ | ++++ | ++++ | ++± | 0 |
| 2. Purified trypsin, 0.2% in the dilute buffer | ++++ | ++++ | 0 | 0 | 0 | 0 |
| 3. Purified chymotrypsin, 0.2% in the dilute buffer | ac | ac | ac | ++± | 0 | 0 |

The mixtures 1, 2, and 3 were kept four hours at 37° C. and overnight at 4° C.

In the same experiment the purified trypsin inactivated more than 50% of a specimen of the Shope papilloma virus-antigen. It seemed to have little or no effect upon sedimentable substances present in extracts of normal and neoplastic rabbit tissues which react with a natural antibody (58).
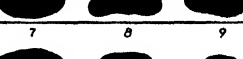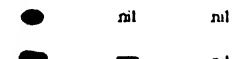
The mixtures containing chymotrypsin were anticomplementary (ac) in the first three tubes, as control mixtures showed, though it was plain that this enzyme also inactivated the Brown-Pearce antigen.

* This had been purified by repeated differential centrifugations and contained 0.310 mgm. N/cc.

growth. For a detailed analysis has shown that regression of the Brown-Pearce carcinoma has been observed more frequently in the absence of the specific antibody than in its presence, while sera procured from hosts in which the tumor had regressed did not suppress the growth of the tumor cells *in vitro* unless they contained the specific antibody (*57*). The observation that the growth regressed abruptly in most of the hosts that developed antibody in high titer, while it frequently grew progressively in hosts that failed to do so, suggests the possibility, among others, that the anti-

stance, which is wholly distinct from the active constituent of the Brown-Pearce carcinoma, and which cannot be detected in extracts of benign papillomas of the type in which the carcinoma originated.

The results of a recent experiment, unreported heretofore, provide data in this relation, and in addition illustrate certain other findings of interest to those concerned with the sedimentable constituents of normal and neoplastic tissue cells. It will be seen in Table 7 that the sera of 12 rabbits carrying large V2 carcinomas all had antibodies that reacted in high titer with a substance present in an extract

| Materials implanted intramuscularly | Titer of specific Brown-Pearce antibody | Outcome of implantations |
| --- | --- | --- |
| Brown-Pearce tumor cells plus | | Tumors excised at necropsy on the 15th day |
| | | *Test rabbits* 1 2 3 |
| a) Locke's solution | | |
| b) Serum from normal control rabbit 7-89 | 0 | |
| c)    "    " Brown-Pearce tumor rabbit 5-47 | 1 128 | nil   nil   nil |
| d)    "    " V2 carcinoma rabbit 21-50 | 0 | |
| | | 4 5 6 |
| e) Locke's solution | | |
| f) Serum from Brown-Pearce tumor rabbit 5-53 | 1 256 | nil   nil   nil |
| g)    "    " V2 carcinoma rabbit 20-52 | 0 | |
| h)    "    " Sarcoma I rabbit 9-41 | 0 | |
| | | 7 8 9 |
| i) Serum from normal control rabbit 7-90 | 0 | |
| j)    "    " Brown-Pearce tumor rabbit 5-51 | 1 256 | nil   nil |
| k)    "    "    "    "    " 5-52 | 1 256 | nil |
| l)    "    " Sarcoma I rabbit 9-40 | 0 | |

The mixtures were kept at 37°C for 2 hours prior to implantation

FIG. 1. Effect of specific antibody on Brown-Pearce tumor cells.
Reprinted from *J. Exp. Med.* 83: 227-250. 1946.

body may have influenced the outcome when it was present—an implication that was considered in detail in a preceding paper (*76*).

## A Distinctive Constituent of V2 Carcinoma Cells

The unique character of the distinctive constituent of Brown-Pearce carcinoma cells was pointed out at the time of its discovery several years ago, along with a discussion of the difficulties inherent in the study of tumors by serological means (*54, 55*). Since then, another transplanted rabbit cancer of unknown cause—the V2 carcinoma (*60*) —has yielded a specific serologically active sub-

of the V2 carcinoma and absent from the extract of a papilloma of the kind in which the carcinoma originated. Eight of the 12 sera reacted also in the lower dilutions with an antigen made from normal rabbit spleen, and seven of these also reacted, though slightly less well in general, with an antigen made from the Brown-Pearce carcinoma. Likewise, the sera of 8 rabbits that had been implanted with Brown-Pearce carcinoma cells all reacted in high titer with a substance present in an extract of the Brown-Pearce tumor, while six of these specimens gave cross reactions in the lower dilutions with the normal spleen and V2 carcinoma antigens.

For quite a long while cross reactions such as

TABLE 7. COMPLEMENT FIXATION TESTS WITH SERA OF RABBITS IMPLANTED WITH BROWN-PEARCE AND V2 CARCINOMA CELLS, RESPECTIVELY

Upper table — antigens: Brown-Pearce Carcinoma Antigen, 1:40 (Serum Dilution); Normal Rabbit Spleen Antigen, 1:40

| Source of Serum | Rabbit No. | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
|---|---|---|---|---|---|---|---|---|---|
| Normal control rabbits | A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | B | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rabbits implanted with the V2 carcinoma | 1684 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1570 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1566 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1820 | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1569 | +++ | ++ | ±0 | 0 | 0 | 0 | 0 | 0 |
| | 1565 | +++ | +++ | +0 | 0 | 0 | 0 | 0 | 0 |
| | 1681 | +++ | + | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1685 | +++ | +++ | ±+ | ± | + | 0 | 0 | 0 |
| | 1682 | +++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | 1733 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 1734 | +++ | +++ | +++ | ++++ | +++ | +++ | +++ | ++ |
| | 1736 | +++ | +++ | ++++ | ++++ | + | +++ | + | + |
| Rabbits implanted with the Brown-Pearce carcinoma | 15-51 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 15-53 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 14-97 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 15-01 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 15-05 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 15-07 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 15-17 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 15-20 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | + |

Lower table — antigens: V2 Carcinoma Antigen, 1:40; D.R. Papilloma Antigen, 1:40

| Source of Serum | Rabbit No. | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
|---|---|---|---|---|---|---|---|
| Normal control rabbits | A | 0 | 0 | 0 | 0 | 0 | 0 |
| | B | 0 | 0 | 0 | 0 | 0 | 0 |
| Rabbits implanted with the V2 carcinoma (same as above) | 1684 | +++ | +++ | +++ | +++ | +++ | 0 |
| | 1570 | +++ | +++ | +++ | +++ | ±+ | ++ |
| | 1566 | +++ | +++ | +++ | +++ | ±+ | 0 |
| | 1820 | +++ | +++ | +++ | +++ | +++ | ++++ |
| | 1569 | +++ | +++ | +++ | +++ | +++ | +++ |
| | 1565 | +++ | +++ | +++ | +++ | +++ | ++ |
| | 1681 | +++ | +++ | +++ | ++ | ++ | +++ |
| | 1685 | +++ | +++ | +++ | ++ | ++ | +++ |
| | 1682 | +++ | +++ | +++ | ++ | ++ | ++ |
| | 1733 | +++ | +++ | +++ | ++ | +++ | +++ |
| | 1734 | +++ | +++ | +++ | | | |
| | 1736 | +++ | +++ | +++ | | | |
| Rabbits implanted with the Brown-Pearce carcinoma (same as above) | 15-51 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 15-53 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 14-97 | +++ | +++ | 0 | 0 | 0 | 0 |
| | 15-01 | ++ | ± | 0 | 0 | 0 | 0 |
| | 15-05 | +++ | + | 0 | 0 | 0 | 0 |
| | 15-07 | ± | ++ | 0 | 0 | 0 | 0 |
| | 15-17 | +++ | 0 | 0 | 0 | 0 | 0 |
| | 15-20 | +++ | 0 | 0 | 0 | 0 | 0 |

these with normal and heterologous neoplastic tissue antigens proved perplexing, but finally it became clear that they are due to antibodies which appear not infrequently in the blood of rabbits carrying transplanted cancers of various sorts and which react with sedimentable constituents of various normal and neoplastic cells (36). The antibodies responsible for these cross reactions have no kinship

any importance in the neoplastic activities thereof —except perhaps those provided by numerous experiments made several years ago in collaboration with Dr. Peyton Rous, in all of which cell-free extracts of the V2 carcinoma failed to produce lesions when brought into contact with normal and altered epidermal tissues of rabbits (60). It is plain, however, that the distinctive V2 substance has no sero-



n = nil, LF, RF, LAT, RAT = left and right forelegs and left and right anterior thigh muscles, respectively.
The hatched tumors later disappeared.

FIG. 2. Results of implantation of Brown-Pearce tumor cells into immunized rabbits.
Reprinted from *J. Exp. Med.* 83: 227–250. 1946.

with the antibody that reacts specifically with the distinctive substance of the Brown-Pearce carcinoma cell, as detailed studies have shown (76), and probably none with the antibody that reacts with the distinctive material of the V2 carcinoma, as is indicated by the findings with serum specimens 1684, 1570, 1566, and 1820 of Table 7.

We are only now learning about the serologically identifiable constituent of the V2 carcinoma cell, and no facts are at hand to indicate whether it has

logical relationship with the rabbit papilloma virus: the antibody which reacts with it has no affinity for the papilloma virus, as many experiments have shown, while the specific antibody for the virus will not react at all with extracts of the V2 carcinoma. It seems especially significant that the antibody for the distinctive constituent of the V2 carcinoma has regularly failed to react with extracts of benign rabbit papillomas of the type in which the carcinoma originated—this as providing an indication perhaps

that the distinctive constituent of the malignant cell may be absent from its benign prototype.

## DISCUSSION[2]

What can be said about distinctive substances such as those just described in relation to the malignancy of the tumor cells that harbor them? Before attempting to answer this question it might be well to look first at certain peculiarities of tumor cells and to survey the results of previous attempts to discern their cause.

### Distinguishing Traits of Malignant Cells

*Autonomy* and *anaplasia* constitute the distinguishing biological characters of cancer cells, as more than a century of experience has now abundantly shown. The former word can be found in practically every definition of cancer: it means that the cancer cell determines its own activities, largely irrespective of the laws of its host organism; indeed the cells of cancers quite regularly compete harmfully with one another, so heedless of the common good is their proclivity for self-regulation. The word *anaplasia* is at home largely in the minds and literature of pathologists: it was coined half a century ago by von Hansemann and is used to define the lack of perfect form and function exhibited by the cancer cell in comparison with its normal fellows; it refers to the alterations that the morbid histologist sees when he looks at cancer cells, and to their lack of proper behavior and its consequences, which the biologist studies.[3]

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[2] The reader should perhaps be forewarned that I shall take advantage in places of the invitation of our sponsors to informality.

It will have occurred to him before now that percipience is required to discern a relationship between the main theme of this Symposium—Heredity and Variation in Microorganisms—and the subjects I shall discuss. Yet on a previous and notable occasion biologists were urged to study the habits of bacteria if they would be wise about the ways of cells (Professor Oswald T. Avery: Presidential Address to the Forty-fourth Annual Meeting of the Society of American Bacteriologists, Baltimore, 1941, unpublished), and it is not inconceivable that the phenomena of bacterial variation may prove to have a relationship in principle with the hereditary variation in tissue cells which constitutes cancer.

[3] He should, though, be called a pathologist when he does this, for he has become a student of disease—the literal meaning of the word. Indeed, cancer is an extraordinary disease. It was not without purpose that MacCallum placed the section on tumors at the end of his *Textbook of Pathology.* For, as he said: ". . . we are quite familiar with the effects of every sort of injurious influence in disturbing temporarily the normal growth of tissue, and can formulate general laws for these effects which are found to be always respected. But tumors do not respect them at all. For that reason I have written of tumor growth separately from all the rest of

The deterioration of form and function in cancer cells finds ready illustration in the materials scrutinized daily by the pathologist. Thus epidermal cells that have become highly malignant hang loosely together in useless and disorderly syncytial masses that invade the contiguous normal tissues, the elements themselves exhibiting irregularities in size, shape, and staining characteristics while failing, more often than not, to mature into keratinized squames and lacking the parakeratotic granules that become conspicuous in the cytoplasm of their normal prototypes upon maturation. Tumor cells arising from the epithelium of ducts are no longer regimented into functioning conduits, however much their arrangement may suggest strivings towards this end; and cancerous cells of glandular origin cannot be counted upon to supply the exocrine or endocrine secretions that the body requires from their unchanged fellows, though often enough they pour these out, sometimes in harmful excess and again only halfheartedly, as if their main activities were directed otherwise. Furthermore, a number of important enzyme activities are either lost or sharply curtailed in cancer cells, as recent studies have shown (*42, 26*)—the findings constituting, it would seem, biochemical manifestations of anaplasia.

The loss of cellular characters—"de-differentiation" to the morphologist—is often referred to as a reversion of malignant cells to the embryonic state, but this is incorrect: embryonic cells, even in their most undifferentiated forms, exhibit an exquisite responsiveness to a whole series of stimuli and controls provided by the organism and by one another, and what is more they manifest an extreme plasticity, being able to differentiate into a wide variety of predetermined forms; de-differentiated cancer cells, on the contrary, respond but poorly or not at all to organismal stimuli and controls, and their potentialities for divergent differentiation are markedly restricted. It is as if some new and compelling factor or influence had developed within the malignant cell which had assumed direction of its activities and crowded out or suppressed the factors responsible for its habitual forms, functions, and potentialities—a possibility that will receive consideration further on. Tumor cells, however, are not completely anarchistic and the masses they form are not actually chaotic,

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pathology as a thing apart, not to be dealt with according to the laws of reaction of normal tissues." In this relation Foulds, an experimental pathologist, has set down his ideas as follows (*35*): ". . . It is far from certain that complete knowledge of the forces which control the development and maintenance of normal structure would solve the problems of cancer . . ."—a point of view, incidentally, that might well receive further consideration from current coordinators of cancer research.

though one frequently stumbles over statements to the contrary in the essays of amateur oncologists. Indeed, an elaborate organization of structure and function is quite consistent with malignancy, the autonomous growth of tumor cells in many instances resembling more or less closely the growth of the normal tissue of origin; while tumor cells may respond in part to the influences that control normal cells, as numerous facts indicate.

Gratuitous mention should perhaps be made of the proliferative capabilities of tumor cells. Second-year students of medicine—those overworked unfortunates of our day who have no time to think about cancer but are often impelled to speak or write about it—not infrequently quote from badly written textbooks the bald sentiment that cancer cells proliferate far more rapidly than do normal cells. This is true, of course, in the sense that the cells of tumors usually proliferate more rapidly than do those of the resting adult tissues round about them. But the more observing and reflective students eventually tumble to the fact that the mass of a primary cancer—a scirrhous carcinoma of the breast, say, or a squamous cell carcinoma of the skin, or even a lymphosarcoma—after perhaps years of growth rarely equals that of a fetus at term, which they know has grown from a single normal cell in ten lunar months; while the book-boring students in due course read that the rate of growth of normal cells may far exceed that of malignant cells of the same type: it is well known for example that the antlers of deers and the claws of fiddler crabs grow at a prodigious rate and probably more rapidly than many tumors do; while normal regenerating liver cells grow much more rapidly than hepatoma cells, as is indicated by their more rapid uptake of radioactive phosphorus (*10*); and cultures of normal adult tissue cells, once they have overcome an initial inertia, may grow quite as rapidly as malignant cells, as direct comparisons have made plain (*95, 49*). From all of which it follows that autonomous growth, not rapid growth, characterizes malignancy.[4]

[4] The distinction has a practical implication for research in the chemotherapy of cancer. For if the life of an organism depends upon the continued proliferation of normal tissue cells (hemopoietic tissues, for example) at a rate as rapid as, or more rapid than, that of cancer cells, then it becomes obvious that chemical or physical agents directed against rapidly proliferating cells as such may well do more harm than good. This has proved true more than once when chemicals such as colchicine and benzol have been given to individuals with cancer; while the principle holds good in the cases of radioactive phosphorus and generalized radiation, and it may prove applicable also to the use of the nitrogen mustards, which are known to produce noteworthy effects in a wide variety of proliferating normal tissue cells. Much more could be said about the lack of specificity of these and other agents sometimes employed in the therapy of cancer, and about the temporary character of the effects they produce, in relation to the autonomy of cancer cells.

This brief survey of the characteristics of malignant cells would be incomplete (and unclassical) without pointed reference to the fact that the change to malignancy is permanent, irreversible, and hereditary. Cancer cells remain such until they die. Furthermore, they never turn back en masse towards the benign or normal state—a principle that is not notably weakened by the considerable spread in histological characteristics manifested by tumor cells of a single type growing in different situations, as exemplified most extremely perhaps by the well-recognized ability of pluripotent teratoma cells to differentiate in more than one direction and by the occurrence of differentiated cells in certain neuroblastomas (*23*). Indeed, the individual peculiarities of form and function in the generality of tumor cells are passed on with remarkable fidelity from daughter cell to daughter cell, in many cases during decades, as studies with the transplanted cancers have abundantly shown, though the cells of these growths too may sometimes de-differentiate further and perhaps stepwise in the direction of increasing malignancy.

*Genetic, Cytological, and Biochemical Methods in the Search for Causes of Cancer*

The conceptions of autonomy and anaplasia and the factual bases upon which they stand have led the majority of cancer workers to the rather obvious assumption that the secret of malignancy lies within the malignant cell. Yet meager indeed have been the results thus far of attempts to bare this secret; though gratuitous ideas about its nature are plentiful enough, as several examples will illustrate.

All too frequently during the past several years attempts have been made to explain away the cause of cancer with the euphonic and somewhat mystical term *somatic mutation*. If by this it is meant that one or more of the genes of a somatic cell have undergone an alteration which brings about the change to cancer and proves hereditary, then the term designates a hypothesis that must remain untestable by Mendelian methods, for the reason that cancer cells proliferate only asexually; while furthermore it is a hypothesis against which formidable theoretical objections can be raised (*46, 72, 73, 81, 93, 79*). If the term is intended to describe the obvious fact that the profound and irreversible cancerous change is passed on from one daughter cell to the next, then it is pretentious as a descriptive term and carries a connotation that may prove false. In either case it provides an enlightening example of how labels lull unwary minds and stifle precision in thought. These remarks, which are meant to be as devastating as I can make them, are not intended to deny the possibility that significant nuclear abnormalities may exist in the cancer cell; their aim is to point to the need of good ideas and effective tools with which to seek out and define such

changes as may actually exist.

The morphological observations of Koller (*61*) and those of Caspersson and Santesson (*14*) provide abundant confirmation of the older observations of Pianese and McCarty and others that cancer cells, like rapidly dividing embryonic cells and those of regenerating tissues, have prominent nucleoli; and they may be significant as providing indications about the nature of the cyclic changes and nuclear-cytoplasmic relationships associated with cellular proliferation; but they provide no discernible basis for an understanding of the autonomy of cancer cells. The conspicuous aberrations of nuclei and chromosomes in cancer cells, so impressive long ago to Pianese and von Hansemann and later to Boveri, and since found to be frequent in some types of cancer cells as not in others, are more or less readily duplicated experimentally in nonmalignant cells (*75*) and are largely discounted nowadays as probably representing secondary manifestations of intracellular disease (*66*); while the more subtle alterations to be seen in the cytoplasm of cultured cancer cells have been deemed by an experienced cytologist more significant than the chromosomal and nuclear deviations visible in them (*66, 67*). In sum it can be said that perseverant and modern cytological studies have failed thus far to reveal within cancer cells any structure that makes them such (*22, 75*), to say nothing of why each is the particular cancer cell that it is; while chemical and metabolic studies have likewise failed to disclose enzymes or other substances distinctive for cancer (*42*) or specific alterations in energy metabolism generally deemed causative of malignancy (*6, 12, 26*)—this in spite of the imaginative promise of Warburg's work of twenty years ago (*107*).

## Biological Studies on Cell-Free Agents as Causes of Cancer

The search for causes of malignancy has not been wholly barren, however: more than thirty years ago the fact was discovered that filtrable and desiccable agents can be separated from the cells of certain avian sarcomas and related growths which are capable of transforming the cells of normal fowl mesenchyme forthwith into malignant cells precisely like those providing them (*87, 88, 89, 30-33, 21, 44, 83*), and more recently other workers have procured from the milk and tissues of a strain of inbred mice a filtrable substance which, in concert with certain hormonal and constitutional influences, plays a crucial part in the etiology of one kind of mammary adenocarcinoma in mice (*70, 62, 63, 7, 8, 1*). Yet it should be emphasized that the nature of the filtrable agents responsible for the tumors mentioned has not been fully elucidated, while next to nothing is known about the ways in which they work their wonders in cells.

The agents that bring about malignancy in fowl cells, for example, are transmitted from one host animal to the next by means undiscerned at present; they are associated with, or concur with, cellular constituents that are identical, so far as can be told, with those of normal fowl cells (*44, 34, 52, 15-19*); yet their precise nature and cellular functions remain undisclosed. The mammary carcinoma agent can pass from teat to mouth during suckling, but it is harmless to mice of many strains and to males and castrated females of susceptible lines, unless hormonal stimulation is given artificially to them during long periods of time. It does not cause cancer directly, and no evidence exists to indicate that it produces *per se* the malignant change which finally comes about in the benign adenomas of long-stimulated mammary epithelium, or that it is essential to the continued proliferation of the malignant cells. Furthermore its physical, chemical, and antigenic properties, as defined thus far (*41*), do not suffice to distinguish it as a virus, the sedimentable constituents of normal tissue cells being similar to it in the properties deemed significant (*58*). It seems premature to attempt to decide at the present time whether the agents responsible for fowl sarcomas and that implicated in the etiology of the mouse mammary adenocarcinoma should be classified as viruses—as much because of the prevailing uncertainties about the nature of the latter (*9, 86, 101, 64, 13*) as because the tumor agents are atypical of the generality of viruses in one or more respects (*e.g.*, in lacking antigenicity within the susceptible species, in lacking infectivity under natural conditions, or in lacking definitive pathogenicity). It would also seem premature, for manifold reasons, to attempt to say now whether the*  filtrable agents extractable from tumor cells have evolved from things intrinsic or extrinsic to the cells susceptible to their action. Parenthetically it may be added that this last holds true as well for the serologically active constituents of tumor cells described in the present paper, though the fact may be significant in this relation that they, like the agents responsible for fowl sarcomas, seem to be associated with, or to concur with, formed cellular constituents identical with those of normal tissue cells (*58, 76*).

The examples just cited are enlightening and important, for they provide the bulk if not the sum of what little we know today about demonstrable factors in the continuing causation of cancer. (The so-called carcinogenic agents—hydrocarbons, X-rays, sunlight, dietary factors, etc.—can be dismissed from consideration in this relation; for while they are important in the genesis of cancer they play no part in its continuation, as is well known.) Yet the fact deserves laboring at this point that the tumors which provide causative agents separable from their cells are actually limited to the small group of naturally occurring fowl sarcomas and allied growths previously referred to. Reasons have

already been given for doubting whether the milk factor does more than cause benign adenomas in the breasts of mice, while even this accomplishment requires the aid of estrogenic stimulation and constitutional susceptibility. Similar arguments can be applied against the Shope papilloma virus as an immediate and continuing cause of cancer: it causes benign papillomas in rabbits and these almost regularly become malignant, but there is no proof that the virus itself is responsible for the enduring malignancy, even though it persisted in two cancers that originated from benign virus-induced papillomas (60, 56), and in spite of the remarkable effects it produces in tarred epidermis (91, 92, 59). Indeed much reflection—centering largely around the fact, recited in this paper, that a distinctive substance can be identified with V2 carcinoma cells which is not detectable in extracts of benign virus-induced papillomas of the sort from which they originated—has made it seem to me probable that this constituent rather than the papilloma virus or an antigenic variant of it may be the continuing cause of the malignancy. Limitations can also be discerned in certain other data commonly considered in this relation. The agent implicated in the etiology of renal carcinomatosis in leopard frogs withstands desiccation and glycerination but its other properties have not been defined (74), while it too acts only after a long interval and sporadically; no evidence exists that it is the continuing cause of malignancy. Finally, the claims of Taylor that filtrable cancer-producing agents can be got from mammalian tumors grown in developing chick embryos (104, 105) have not been confirmed in other laboratories (11, 106); hence they would seem to be undeserving of further consideration on the basis of the data at hand.

## Serological Studies and the Problem of Cancer Causation

What can be learned about the continuing causation of malignancy in the vast generality of cancer cells which have not thus far yielded causative agents to the numerous and artful experiments of seasoned workers? The mere repetition of attempts to extract a causative agent directly from each new growth that comes within the ken of cancer investigators is apt to result in failure after failure if ordinary methods are employed, as some know all too well. Indeed, the protagonists of the view that cancers are caused by extrinsic viruses have forearmed themselves against such an outcome by elaborate formulations of the notion that latent viruses (2), or "dead-end," masked, or mutant viruses—hypothetical agents, that is to say, which are no longer infectious for new cells though pathogenic for the neoplastic ones they inhabit (90)—may be immediately responsible for malignancy in the generality of tumors. Whatever may be the

merits of these hypotheses, it is obvious that new methods must be applied if the continuing causes of malignancy are to be demonstrated directly in the generality of cancer cells. Will those of serology be helpful in this relation?

Such was the question that led more than ten years ago to the discovery of the distinctive constituent of the Brown-Pearce carcinoma cells, and to the later developments surrounding this material, as reviewed in the present paper. In this relation it may be pointed out that the distinctive substance is a constant and prominent constituent of Brown-Pearce tumor cells, having been recoverable regularly and in large amounts from them during the past ten years; obviously it increases in amount as the tumor cells proliferate, though whether autocatalytically or otherwise is anybody's guess. It is like the only actuating causes of cancer now known—the fowl sarcoma agents—in having a rigid specificity (being confined, that is, to cancer cells of one particular kind, though, as already mentioned, the possibility cannot be ruled out that the substance might exist in comparatively small amounts in normal tissue cells, and remain undetectable by our methods), in being labile to heat and to changes in pH and in having other properties which suggest that it is probably a protein and perhaps a ribose nucleoprotein, and—as likewise previously mentioned—in being associated with, or concurring with, constituents similar in chemical and serological properties to the microsomes of normal tissue cells as defined by Claude (76). The latter consideration seems important as perhaps identifying the distinctive Brown-Pearce constituent with the cytoplasm, particularly in view of the implication that formed constituents of the cytoplasm may play important parts in the activities of cells, as for example in determining differentiation (108, 43) and in mediating synthetic activities as well perhaps as energy metabolism (102, 50). The Brown-Pearce substance differs from the filtrable tumor-producing agents in that it lacks pathogenicity for other cells—or, to state this more precisely, the Brown-Pearce material failed to manifest pathogenicity in the tests made several years ago—though indirect evidence that it may play an important part in the proliferative activities of the tumor cells with which it is naturally associated is provided by the recent finding that the growth of the tumor cells is suppressed by the antibody which reacts specifically with their distinctive constituent (57).

It should be mentioned in passing that the principle has long been apprehended in immunology that antibodies may interfere with the proliferative capabilities of cells. Ascoli (3) and Dochez and Avery (29) for example have studied the "antiblastic" effects of certain antibacterial antibodies, and Taliaferro (103) has described "ablastic" antibodies which prevent reproduction of *Trypanosoma*

*lewisi* in the rat and *T. duttoni* in the mouse, an interesting fact being that the parasites seem to remain alive, motile, and capable of infecting new hosts during a sojourn of months in the blood of animals having effective titers of the ablastic antibodies. Recently, Gorer has procured a "protective antibody" by injecting leukemic cells into mice naturally resistant to them, which is capable of retarding the growth of the leukemic cells under appropriate conditions (*39*); and Augustine has provided evidence that antitrypanosomal antibodies are notably lethal for dividing cells (*4*); while Medawar has put forth the hypothesis that the destruction of homologous skin grafts in rabbits is due to the action of antibodies that prevent nuclear division in the cells of the grafted tissue (*80*); and Harrison and Fowler have found that exposure of certain ciliates (tetrahymena of the Colpidium-Glaucoma group) to the action of dilute rabbit antisera results in a remarkable interference with the completion of cellular division, bizarre chains and multinucleated giant cells developing as a result (*47*). Finally, Green has stated in a preliminary report that an immune serum prepared in rabbits against a sedimentable material of mouse mammary adenocarcinoma cells will inhibit the growth of the latter upon incubation therewith *in vitro* (*40*). It may be recalled, as bearing on all this, that one of Ehrlich's great contributions was his theoretical explanation that dyes injure the reproductive power of trypanosomes without affecting their other vital functions, as Sir Henry Dale has pointed out (*24*). It may be significant also, as providing an indication of the nature of the distinctive Brown-Pearce constituent, that its host cells do not "protect" it from the action of its specific antibody, whereas many living cells, notably certain neoplastic ones, provide such protection for viruses (*56*).

The difficulties inherent in the study of tumors by serological methods were discussed several years ago (*54, 55*), as already mentioned, along with a consideration of the possibility that the distinctive constituent of Brown-Pearce carcinoma cells might be responsible for their neoplasia. Since then a number of additional complexities have been encountered in serological work with tumors (*58, 36*), and what may prove to be an important limitation has been disclosed; namely, the fact that antibodies capable of reacting with sedimentable constituents of normal and neoplastic tissue cells, while they appeared frequently in the blood of animals carrying transplanted cancers of various sorts, could not be detected in the sera of a number of animals carrying autochthonous growths (*36*)—a finding which, along with those of Dmochowski (*28*) and of Hoyle (*51*) in other serological studies, may well be pondered by workers who contemplate serological experiments with materials derived from cancerous patients. Yet, as already several times told,

serological methods are now being employed in the study of a second distinctive sedimentable constituent—that identified with the V2 carcinoma cell and apparently absent from its benign prototype. It would seem likely that similar studies with other tumors would yield similar findings, though it is only fair to add that painstaking experimentation, with numerous and adequate controls, would probably be required, and perhaps fortunately chosen materials as well.

Another point deserves consideration in this relation; namely, the possibility that normal tissue cells of various sorts might yield specific constituents similar to those of tumor cells as described herein. In the extensive studies with a natural antibody that reacts with sedimentable constituents of many normal and neoplastic tissue cells, no evidence of organ specificity was encountered (*58*), and much the same was true in more limited tests with induced antibodies having similar affinities, which appeared not infrequently in the blood of rabbits carrying one or another of several transplanted cancers (*36*). In experiments performed by other investigators, sedimentable constituents from various tissues were injected into animals of foreign species with a view to testing for species- and organ-specific reactions. The antisera gave species-determined reactions, as well as reactions against Forssman and Wassermann type antigens, which were far more conspicuous than were any organ or tissue specificities; while antibodies were not demonstrable when the materials were injected into animals of the same species (*37, 48*). In sum, the findings thus far have failed to disclose any evidence that the sedimentable constituents of normal tissue cells exhibit a serological specificity in any way comparable to that shown by each of the distinctive constituents of carcinoma cells described in the present paper. Yet it seems worth while to consider the possibility that serological methods of other sorts might prove helpful in defining in more significant fashion any differences that may exist in the sedimentable constituents of various normal tissue cells.

### THEORETICAL CONSIDERATIONS

While morphological, biochemical, and serological methods may continue to provide additional and perhaps pertinent information in the search for the continuing causes of cancer, it is obvious that the ultimate solution of the problem will depend upon the development of biological methods whereby living normal cells can be transformed into malignant cells by means of materials derived from the latter. And in spite of the practical difficulties and failures encountered heretofore in the repeated attempts to accomplish this, it seems plain that theoretically the autonomy of cancer cells requires within them the presence of something newly activated and distinctive. Furthermore, it is an interesting fact that

this conception has found but scanty expression in the literature of cancer, though it has achieved recognition by at least three seasoned cancer workers. The eldest of these, Leo Loeb, basing his reasoning largely upon histological studies of precancerous lesions and upon the observation that the parenchymal cells of tumors may sometimes stimulate their stroma to neoplasia, with reference also to the implications provided by the embryonic organizers of Speymann, has proffered the following hypothesis (*71, 73*): "... Tentatively it may be assumed that ... a growth substance is produced or increased in quantity and that the production of this substance is renewed autokatalytically. Thus growth·processes of certain cells are intensified irreversibly, and certain cellular, metabolic, and functional processes are altered ...." The youngest, Foulds, reasoning broadly from an astute and critical histological and biological analysis of tumors, wrote the following (*35*): "There is abundant evidence that tumors consist of cells which are permanently altered from the normal. Apparently the properties inherited from their parent tissues by tumor cells do not include those which make them particularly dangerous." Finally, Peyton Rous, impressed by the neoplastic potentialities of embryonic cells and by the difficulties inherent in the supposition that these are conferred by extraneous viruses, has recently inquired (*94, 96*): "... Can it be that animal cells, when brought to the abnormal state in which neoplastic change occurs, elaborate self-reproducing substances which make them into tumor cells and maintain them as such?" In addition, a number of other workers, some of whom possess only theoretical knowledge about cancer, have recently put forth similar formulations (*84, 25, 45, 27, 110*), bolstering these abundantly with references to the knowledge now developing about factors that influence the structure and activities of a wide variety of cells (*65, 85, 97, 98, 99, 5, 77, 78, 68, 69, 100, 109*). It is striking indeed that the hypotheses mentioned are not mutually exclusive, and something of an indictment that all of the substances postulated in them as responsible for malignancy remain at present hypothetical, none having been procured and studied.

Can one assume that distinctive constituents such as those associated with Brown-Pearce and V2 carcinoma cells are present in the generality of cancer cells and responsible for their continuing malignancy, each type of neoplastic cell having a special kind of constituent which, by its intracellular activities, determines the particular misbehavior of the cell (autonomy), and which, by crowding out or overgrowing or suppressing the activities of the cell's normal constituents, brings about a degradation of its normal form and functions (anaplasia)? Do the specific constituents already studied have a relation to the filtrable agents responsible for fowl sarcomas (*89*), to the formed constituents

of cytoplasm (chondriosomes, mitochondria, and the like) which cytologists have long contemplated as possible determinants of cellular differentiation (*108, 43*), and in turn to the microsomes of Claude, which—facts as well as teleology indicate—may have important physiological functions (*19, 50*)? Do they provide concrete examples of "autokatalytic growth substances" such as Loeb has tentatively postulated as the immediate causes of cancer (*71, 72, 73*)? Are they self-reproducing substances of the kind that Rous imagines may be the actuating causes of malignancy (*94, 96, 78*)? What similarities do they show to the cytoplasmic substances now being found to exert such remarkable influences on a wide variety of cells (*65, 85, 97, 98, 99, 5, 77, 78, 68, 69, 100, 109*)? Will they manifest relationships with equally distinctive components yet to be defined in the nuclei of tumor cells?

The questions have more than theoretical and academic interest. For cancer research, like all other endeavors, has its practical aspects, and nowadays these bulk large. A clear understanding of the factors and principles involved in the continuing causation of malignancy is important as providing the basis for an intelligent approach to the problems of cancer prevention and cancer cure. If the autonomy of cancer cells is due in whole or in part to the development within them of distinctive cytoplasmic constituents such as those herein described, then the study of the origin and intracellular functions and relations of these substances becomes important in the problems of cancer causation[5] and cancer prevention; while the search for ways to suppress their action in the living cell, as is apparently accomplished by the antibody that reacts specifically with the distinctive constituent of Brown-Pearce carcinoma cells (*57*), may have implications for cancer control. If the anaplasia of cancer cells comes about because their normal constituents have been crowded out by abnormal ones, then it becomes easier to understand how and why cancer cells fail to respond to the organismal controls that govern their normal fellows, and incidentally why chemical substances that presumably act upon normal cellular constituents (hormones, for example) may theoretically have only limited usefulness in

[5] Is it meaningful in this relation that fully differentiated neurones (anterior horn cells, Betz cells, Purkinje cells, for example), in which the development of Nissl substance may perhaps have crowded out other cytoplasmic constituents, rarely if ever become cancerous? As bearing further perhaps on the pathogenesis of abnormal cytoplasmic constituents in cancer cells, it may be significant that Opie reported only last week (*82*) that butter yellow (dimethylaminoazobenzene) causes degenerative changes in liver cells accompanied by chromatolysis of cytoplasmic structures that stain with basophilic dyes because they contain ribonucleic acid. The chromatolysis is followed by focal regeneration of liver cells which contain ribonucleic acid in their cytoplasm, the basophilic cells of these foci giving rise not infrequently to hepatomas.

cancer therapy. Perhaps the future will disclose whether distinctive constituents such as those herein described are present in the generality of cancer cells, and whether they provide useful materials as well as intellectual provender for those who seek within cancer cells the cause of their enduring malignancy and the means for their control.

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## DISCUSSION

Altenburg: There can no longer be any doubt that the cytoplasm contains bodies, in addition to plastids, which are capable of self-reproduction and mutation. The problem now is, what is the nature of these bodies? One possibility is that they are plasmagenes in one sense or another—either cytoplasmic genes that are independent of the nucleus, or that are intimately related in point of origin to nuclear genes, as postulated especially by Spiegelman and the Lindegrens, or bodies derived, according to Darlington, from a combination of cytoplasmic proteins and nucleic acid. A second possibility, but one which does not necessarily exclude the first, is that the bodies in question are useful intracellular symbionts. These would be analogous to the bacteroids in the fat bodies of cockroaches and in various tissues of other organ-

isms, but they would be ultramicroscopic, and akin to viruses. We might call them *viroids*. (Altenburg, E., Amer. Nat. 80:559-567, 1946.)

It is conceivable that these supposed viroids represent the naked-gene stage in evolution, and that organisms at this stage were at one time free-living (possibly in the Archeozoic), but that they became extinct as free-living organisms soon after the appearance of bacteria or other organisms which had a cellular organization and with which they could not compete as free-living organisms, having in the meantime become symbionts in the one-celled organisms. It might then be expected that viroids become *universal* symbionts, since they would have become symbionts at the one-celled stage in evolution, and might very well have become indispensable constituents of the cell, necessary perhaps for the production of certain vitamins and special classes of enzymes and for many other processes. It might also be expected that from time to time, in the course of evolution, viroids gave rise to mutants. Some of these would be distinguished from the normal viroids by some difference in the type of enzyme that they control, thus giving rise to bodies referred to as "plasmagenes" or "cytogenes."

Sometimes the mutants might have pathological effects, and they would then be referred to as "viruses." A very important class of viruses would be those that happened to cause the continued growth of certain classes of cells, thus causing cancer. But whatever the nature of the mutants, it is to be expected that at first they could not be transmitted, as a rule, to a different host by an insect or other vector, since they might have become incapable of remaining alive, for even the short period necessary for transmission, apart from the living cell to which they had become adapted through a long period of evolution. The cancer viruses would in general belong to this class. However, very exceptionally, viroids might have given rise to viruses which were capable of transmission to other hosts. These very mutants might often have been tumor-inducing viruses of a transmissible sort, as is true, for example, of a certain tumor virus naturally transmissible in cottontail rabbits; and by a further process of mutation and natural selection, they might have evolved into the viruses which cause such diseases as yellow fever, smallpox, etc.

We could thus distinguish between two classes of viruses: (1) *neoviruses* or viruses newly arisen from a viroid, and (2) *paleoviruses* or viruses that arose, perhaps millions of years ago in many cases, from a viroid. Cancer would almost without exception be caused by neoviruses; hence it is not ordinarily transmissible to a new host. Each individual case of cancer would represent a new disease, and would be due to a new mutation. Chemical carcinogens would, on the viroid theory, cause cancer by causing viroids to mutate to neoviruses,

some of which happened to cause continued growth.

Nuclear gene mutations might themselves sometimes act as chemical carcinogens, and in this event the host with the mutant gene would have a hereditary form of cancer. However, such mutant genes are rare in point of origin. Most mutant genes, as a matter of chance, would not cause a viroid to mutate, at least not in such a way as to cause the continued growth of cells. Hence agents which cause an increase in the nuclear gene mutation rate do not necessarily act as carcinogens. On the other hand, chemical carcinogens do not as a rule seem to have any noticeable effect on the nuclear gene mutation rate. This is to be expected from the fact that nuclear genes are protected from external chemical agents by the cytoplasm. By contrast, viroids would be readily accessible to such agents.

Viroids are not to be confused with "latent viruses"; for viroids are supposedly normal and essential constituents of a cell. Latent viruses are not; they are primarily pathogens. Hence one would hardly expect carcinogens to be capable of producing cancer in every normal host, if latent viruses were the cause of cancer, as has been supposed for example by Andrewes. For one would have to suppose (as pointed out by Rous) that every normal individual carried a latent virus for each of the many kinds of cancer that can be induced in normal tissues by carcinogens. But viroids would expectedly be present in all normal cells on the viroid theory; and by random mutation, they might give rise to viruses of many different kinds—some of which might cause continued cell division and growth. Moreover, there is a practical distinction between a viroid and a latent virus, a distinction which would have to be taken into account in the treatment of cancer. For on the latent-virus theory there would not necessarily be any objection to a treatment which killed the predecessor of the cancer virus (the supposed latent virus), since the latent virus is of no value to a cell. But on the viroid theory, the predecessor would be a viroid, and any treatment which killed it would be fatal to the remaining normal cells of the host. It is obvious that, on the viroid theory, any antibiotic used as a cancer treatment might have to possess a high degree of specificity to distinguish between viroid and neovirus.

The viroid theory explains the fact that certain bodies have the properties not only of normal cell constituents but also of independent organisms. For viroids would have a certain capacity for evolution independently of their host, since they are separate organisms. They could, therefore, presumably give rise (through mutation and natural selection) to viruses which could live for a while in an intermediate host very different from the principal host. Thus they might become transmissible by an insect vector. This would hardly be

expected of a gene which belonged to the host cell, since genes (and the enzymes they produce) are parts of a complicated close-knit organization. This difference in capacity for transmission constitutes a real distinction between a viroid and a plasmagene, if by the latter we mean a body that is part of the gene system of the host cell. A viroid is therefore not simply a plasmagene with another name; and the possibility that viroids exist will have to be taken into account in any discussion of inheritance involving cytoplasmic bodies that are ultramicroscopic in size.

# NOVEL GENOTYPES IN MIXED CULTURES OF BIOCHEMICAL MUTANTS OF BACTERIA

## JOSHUA LEDERBERG[1] AND E. L. TATUM

Hershey has reported (1) the occurrence of novel combinations of inherited characters in a bacterial virus. It may not be amiss to describe briefly some experimental fragments, relating to a situation in the bacterium *Escherichia coli*, which may be similar in some respects.

Tatum, in reviewing biochemical mutations in *E. coli* (6), has pointed out the advantages offered by these characters for genetic analysis. In particular one may note the facility and certainty with which may be detected readily by plating heavy suspensions of the washed cells into a minimal agar medium, in which only the prototrophs will form macroscopic colonies. Their frequency is very much greater than that anticipated on the hypothesis that the prototrophs result from the coincidental occurrence, in the same clone, of reversions of two or more "loci." Furthermore, single cultures of the same multiple mutants grown and tested under comparable conditions have not been found to

TABLE 1. TYPES ISOLATED FROM SINGLE AND MIXED CULTURES. MUTANTS USED ARE INDICATED ON THE LETTERED LINES.

| From single and mixed | From mixed only | From single and mixed |
|---|---|---|
| A........ $B^-M^-P^+T^+$ ...... and ...... $B^+M^+P^-T^-$ | | |
| $B^+M^-P^+T^+$ $B^-M^+P^+T^+$ | $B^+M^+P^+T^+$ * | $B^+M^+P^+T^-$ $B^+M^+P^-T^+$ |
| B........ $B^-M^-P^+T^+R$ ...... and ...... $B^+M^+P^-T^-$ | | |
| ** | $B^+M^+P^+T^+R$ * $B^+M^+P^+T^+$ * | ** |
| C........ $B^-M^-P^+T^+$ ...... and ...... $B^+M^+P^-T^-R$ | | |
| ** | $B^+M^+P^+T^+R$ * $B^+M^+P^+T^+$ * | ** |
| D........ $B^-M^-P^+T^+R$ ...... and ...... $B^+M^+P^-T^-R$ | | |
| ** | $B^+M^+P^+T^+R$ * | ** |
| E........ $B^-\Phi^-C^+P^+T^+$ ...... and ...... $B^+\Phi^+C^+P^-T^-$ | | |
| $B^-\Phi^-C^+P^+T^+$ $B^-\Phi^+C^-P^+T^+$ $B^+\Phi^-C^-P^+T^+$ | $B^+\Phi^+C^+P^+T^+$ * $B^-\Phi^+C^+P^+T^+$ $B^-\Phi^+C^+P^-T^+$ | $B^+\Phi^+C^+P^-T^+$ $B^+\Phi^+C^+P^+T^-$ |

\* Prototroph.
\*\* See A for biochemical variations.
The letters refer to requirements for essential metabolites as follows:

| | |
|---|---|
| B = biotin | M = methionine |
| Φ = phenylalanine | P = proline |
| C = cystine | T = threonine |
| R = Resistance to virus T1. | |

they can be classified, and their relative stability and independence. Since many of the single biochemical mutants of *E. coli* revert with a measurable frequency of the order of $10^{-7}$ (3, 5) we have used the multiple mutants, obtained by iterated mutations, referred to previously (6).

When multiple mutants are grown in mixed cultures in complete (yeast extract-peptone-glucose) medium, there have repeatedly appeared appreciable numbers of prototrophic (4), or nutritionally wild-type, cells. Although these have never been found, thus far, in a proportion higher than $10^{-7}$, they

contain prototrophs, although small numbers of cells reverted at a single "locus" are, of course, found.

Since it has been established in this laboratory that different biochemical mutants are capable of supplying each others' growth-factor requirements by exchange through the medium, it would appear to be possible that these prototrophs represent heterogeneous aggregations of the different mutants. Attempts to detect or induce the segregation of the components of such putative aggregates by biological and physical means have, as indicated below, been uniformly unsuccessful. Therefore it seems likely that the prototrophs are genotypically unique cells.

After having passed through a single colony isolation, the prototrophs are quite stable; and of many hundreds of colonies isolated from cultures grown on complete medium, all have proved prototrophic.

Cultures of a prototroph, grown in complete medium, were irradiated with ultraviolet light of such dosage that the number of colonies which appeared on plating in complete agar medium was reduced to $1:10^5$. At this rate of killing, it is evident that most of the supposed aggregates had no surviving representatives. Of the remainder it is likely that only a single cell would survive, in most instances, to form a colony. Nevertheless, each of several hundred colonies that were tested were prototrophic.

Fortunately, the strain with which these experiments were conducted (K-12) is susceptible to the bacterial virus T1 (2). In the following experiment multiple mutant strains were used which required biotin and methionine $(B^-M^-P^+T^+)$ and threonine and proline $(B^+M^+P^-T^-)$ respectively. In addition, mutations, occurring spontaneously at a low rate, for resistance (R) to T1 were selected for by the procedure described by Luria and Delbrück (2).

Mixed cultures were plated out as before, and prototrophs isolated and tested for virus resistance. When mixtures of susceptible strains were studied, only susceptible prototrophs were found. Similarly, when resistant strains were mixed the prototrophs obtained were exclusively resistant. When, however, a mixture of $(B^-M^-P^+T^+R)$ and $(B^+M^+P^-T^-)$ was used, of ten isolated prototrophs, 8 were resistant and 2 sensitive. When $(B^-M^-P^+T^+)$ and $(B^+M^+P^-T^-R)$ were used, 3 were resistant and 7 sensitive. If the prototrophs consisted of aggregates of the original mutants,

each prototroph culture in these cases should have had a large proportion of resistant cells. Yet we have mentioned above the finding of 9 prototroph cultures which were completely lysed by T1. The occurrence of both resistant and sensitive prototrophs is evidence, also, for their internal homogeneity, as one might expect either resistance or susceptibility to be dominant (see Table 1).

Combinations of other mutants have given rise to prototrophs. In particular, isolations have been made from a mixture of proline-threonineless $(B^+\Phi^+C^+P^-T^-)$ and biotin-phenylalanine-cystineless $(B^-\Phi^-C^-P^+T^+)$ plated into agar containing biotin, phenylalanine and proline. In addition to prototrophs and single reversion types, a biotinless $(B^-\Phi^+C^+P^+T^+)$ and a biotin-prolineless $(B^-\Phi^+C^+P^-T^+)$ have been isolated.

*Note added in proof:* Additional experiments and an interpretation of these data have been reported by us. ("Gene recombination in *Escherichia coli.*" Nature 158: 558. 1946.)

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# THE CYTOGENE THEORY[1]

## CARL C. LINDEGREN AND GERTRUDE LINDEGREN

Genetical experiments have shown that the gene is a duality, with both chromosomal and cytoplasmic components. The present paper reports experiments supporting this view. We will also discuss the cytology of the yeast cell. This cell is a primitively compartmented structure with organelles arranged in a manner strikingly different from that with which we are acquainted in the cells of higher forms. In the yeast cell the chromosomes sometimes contain large quantities of volutin (21). Rafalko (22) has recently shown that they are also faintly Feulgen-positive, indicating the presence of some desoxyribose nucleoprotein. Desoxyribose nucleoprotein, however, is found in abundance in the centriole of the yeast cell, and this structure may be the equivalent of heterochromatin in higher organisms. Ribose nucleoprotein is present in granules in the cytoplasm, and these may be the equivalent of the nucleolus.

## MENDELIAN AND NONMENDELIAN SEGREGATIONS

### Tetrad Analysis in Saccharomyces

The ability to ferment galactose is widely distributed in the genus Saccharomyces, and we have had great difficulty in finding a suitable nonfermenting haplophase capable of producing hybrids in which we could study the inheritance of this character. We obtained from Dr. L. J. Wickerham a culture of *Saccharomyces microellipsoideus* (NRRL No. Y-1350), which was a relatively slow fermenter of galactose. A mating was made between a haplophase culture from it and a haplophase culture from our *S. cerevisiae* (FLD-1A), which is a galactose fermenter. One ascospore was isolated from a single ascus of the hybrid and was found to be incapable of fermenting galactose.

The nonfermenter was mated with a haplophase galactose fermenter originating from *S. cerevisiae* (Dr. E. M. Mrak, No. 93) to produce Hybrid I (Table 1). An α-mating-type, galactose-fermenting (αG) culture was mated with the a-mating-type nonfermenter (ag). Thirteen asci were analyzed from the αG × ag hybrid. Hybrid I was demonstrated to be heterozygous for mating type (α/a) and galactose fermentation (G/g).

The haplophase cultures derived from the asci were designated a or α by mating them with standard-mating-type strains derived from Mrak's culture No. 93 of *S. cerevisiae*. Galactose fermentation was tested by inoculating small tubes of nutrient galac-

tose broth containing smaller inverted tubes. In all these and subsequent tests, the sugar solution was sterilized by filtration through a Seitz filter and added to the autoclaved medium to make a 4% solution of the carbohydrate in the finished medium.

TABLE 1

*Pedigree 1:* Genetical analysis of four-spored asci obtained from a succession of hybrids heterozygous for genes affecting mating types and galactose fermentation. The first hybrid was obtained by mating a galactose-fermenting haplophase of *S. cerevisiae* with a nonfermenter of galactose, derived from a hybrid between *S. cerevisiae* and *S. microellipsoideus*.

| Ascus | | Spores | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| *S. cerevisiae* (Mrak 93) | 1 | αG | aG | αG | aG |
| *S. cerevisiae*×*S. microellipsoideus* | 1 | ag | | | |
| Hybrid I | 1 | αg | αg | aG | |
| 93-1C×(*S. cer.*×*S. mi.*) | 2 | αg | aG | αG | ag |
| αG×ag | 3 | ag | αG | ag | αG |
| | 4 | αg | aG | αG | |
| | 5 | ag | αG | αg | aG |
| | 6 | ag | ag | αG | αG |
| | 7 | aG | αG | αg | ag |
| | 8 | ag | αG | ag | αG |
| | 9 | aG | αG | ag | αg |
| | 10 | ag | aG | αg | |
| | 11 | αG | ag | αg | aG |
| | 12 | αG | ag | αG | ag |
| | 13 | αg | aG | αG | ag |
| Hybrid II | 1 | aG | aG | αG | αG |
| (93-1C×Hybrid I-2B) | 2 | aG | aG | αG | |
| αG×aG | 3 | aG | αG | αG | |
| | 4 | αG | aG | aG | |
| | 5 | aG | aG | αG | |
| | 6 | aG | αG | aG | αG |
| | 7 | αG | aG | αG | aG |
| Hybrid III | 1 | aG | aG | αG | αG |
| (93-1C×Hybrid I-1C) | 2 | aG | aG | αG | |
| αG×aG | 3 | aG | αG | αG | aG |
| | 4 | αG | aG | αG | aG |
| | 5 | aG | αG | αG | aG |
| | 6 | aG | αG | aG | αG |
| Hybrid IV | 1 | αG | ag | aG | αg |
| (93-1C×Hybrid I-2D) | 2 | αg | aG | αg | |
| αG×ag | 3 | aG | ag | αg | αG |
| | 4 | aG | αg | ag | αG |
| | 5 | αG | αg | aG | ag |
| | 6 | ag | αG | aG | αg |
| Hybrid V | 1 | αg | ag | ag | ag |
| (*S. cer.*×*S. mi.*-1A× | 2 | ag | αg | ag | |
| Hybrid I-1A) | 3 | αg | ag | ag | ag |
| ag×αg | 4 | αg | ag | ag | ag |
| | 5 | αg | ag | ag | ag |
| | 6 | αg | αg | ag | |
| | 7 | αg | ag | αg | ag |

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Fermentation in the positive cultures was recognizable within 24 or 48 hours, but the negative cultures were not discarded until they had been under observation for more than three weeks.

Hybrid II was produced by mating an $\alpha$ galactose-fermenting culture ($\alpha G$) with an $a$ galactose fermenter ($aG$). Mating type segregated in the 7 asci, but the zygote was homozygous for galactose
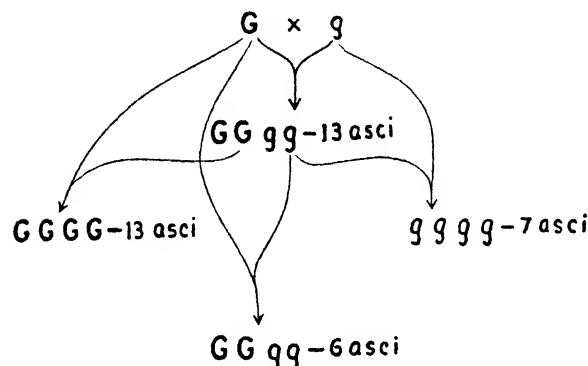


FIG. 1. A diagram summarizing the data presented on the inheritance of galactose-fermenting ability shown in pedigree 1. No exception to standard Mendelian inheritance was found. Thirteen asci were analyzed from a heterozygous hybrid made by mating a galactose fermenter ($G$) by a nonfermenter ($g$); two spores in each of these asci carried the dominant gene controlling fermentation of galactose, and two carried the recessive allele. A backcross of fermenter to the fermenter parent produced thirteen asci; all four spores in each of these asci carried the fermenting gene. A backcross of the nonfermenter to the nonfermenting parent produced seven asci, each of which contained four nonfermenting spores. A heterozygous zygote was produced by backcrossing a nonfermenter to the fermenting parent; six asci were analyzed and each contained two fermenting and two nonfermenting spores. This analysis shows quite convincingly that in some pedigrees the genes controlling fermentation of galactose may behave in a regular Mendelian manner.

fermentation. Hybrid III was also produced by mating an $aG$ with an $\alpha G$ culture. Hybrid IV was made by mating an $\alpha$ galactose fermenter ($\alpha G$) with an $a$ nonfermenter ($ag$). Mating type and galactose fermentation segregated regularly. Hybrid V was made by mating two nonfermenters ($\alpha g \times ag$). Mating type segregated regularly, but the zygote was homozygous for inability to ferment galactose. The data, summarized in Table 1 and Fig. 1, show that mating type and the ability to ferment galactose were transmitted in a regular Mendelian manner.

*Analysis of a hybrid heterozygous for both galactose and melibiose fermentation.* A haplophase of *S. carlsbergensis* (Mrak's culture No. 126) capable of fermenting both melibiose and galactose ($\alpha MG$) was mated to a haplophase ($amg$) incapable of fermenting either melibiose or galactose from pedigree 1. Table 2 shows the pedigree. Three hybrids

of the same genotypic constitution ($\alpha MG/amg$) were formed, and 18 asci were analyzed. Regular segregation occurred in all complete asci except the third ascus in Hybrid I and the first ascus in Hybrid II. In these two asci, more than 2 of the ascospores were fermenters. Seven of the 8 spores in these two asci were $MG$ and one was $mG$, indicat-

TABLE 2

*Pedigree 2:* Genetical analysis of four-spored asci of hybrids heterozygous for genes affecting mating type, galactose fermentation, and melibiose fermentation. The first three hybrids were obtained by mating a haplophase of *S. carlsbergensis* with a nonfermenter from pedigree 1.

| | | Spores | | | |
|---|---|---|---|---|---|
| | Ascus | A | B | C | D |
| *S. carlsbergensis* | 1 | $\alpha MG$ | | | |
| Pedigree 1, Hybrid I | 8 | $amg$ | | | |
| Hybrid I | 1 | $aMg$ | $\alpha MG$ | $\alpha mg$ | $amG$ |
| (carlsbergensis 1A | 2 | $\alpha mG$ | $\alpha MG$ | $aMg$ | $amg$ |
| $\times$8A) | 3 | $\alpha MG$ | $\alpha MG$ | $\alpha MG$ | $\alpha MG$ |
| $\alpha MG \times amg$ | 4 | $\alpha MG$ | $amG$ | $aMg$ | $\alpha mg$ |
| | 5 | $amG$ | $\alpha mG$ | $aMg$ | $aMg$ |
| | 6 | $amG$ | $aMg$ | $amg$ | |
| | 7 | $\alpha mG$ | $\alpha MG$ | $aMg$ | |
| | 8 | $\alpha MG$ | $amG$ | $aMg$ | $amg$ |
| | 9 | $\alpha MG$ | $aMG$ | $\alpha mg$ | $amg$ |
| | 10 | $amg$ | $aMG$ | $\alpha mG$ | $aMg$ |
| | 11 | $aMg$ | $\alpha mg$ | $\alpha MG$ | $amG$ |
| | 12 | $amg$ | $\alpha MG$ | $aMg$ | |
| Hybrid II | 1 | $\alpha MG$ | $aMG$ | $amG$ | $aMG$ |
| Pedigree 1, Hybrid V | 2 | $amg$ | $\alpha mG$ | $aMG$ | $\alpha Mg$ |
| 2C$\times$carlsbergensis 1A | 3 | $\alpha mg$ | $amG$ | $aMG$ | $aMg$ |
| $amg \times \alpha MG$ | 4 | $amg$ | $\alpha mG$ | $aMG$ | $aMg$ |
| Hybrid III | 1 | $\alpha mg$ | $aMG$ | $\alpha mg$ | $aMG$ |
| Pedigree 2, Hybrid I, | 2 | $aMg$ | $amG$ | $amg$ | $\alpha Mg$ |
| 2B$\times$Pedigree 1, Hybrid I, 3A | | | | | |
| $\alpha MG \times amg$ | | | | | |
| Hybrid IV | 1 | $amg$ | $\alpha mg$ | $\alpha mg$ | $amg$ |
| Pedigree 2, Hybrid I, | 2 | $amg$ | $amg$ | $amg$ | $amg$ |
| 1C$\times$Pedigree 1, Hybrid IV, 1B | | | | | |
| $amg \times amg$ | | | | | |

ing that some special, possibly local condition differentiated these two asci from the others, in which regular Mendelian ratios for both genes were found. Analysis of the asci in which regular ratios were found showed that the *S. carlsbergensis* haplophase contained one gene controlling fermentation of galactose and one gene controlling fermentation of melibiose.

Hybrid IV was produced by mating a nonfermenter of both sugars with a similar individual ($amg \times amg$), and all 8 progenies failed to ferment either melibiose or galactose.

### "Masked" Recessives

The haplophase culture of *S. carlsbergensis* used as a mating parent in pedigree 2 (Table 2) is the

same culture which we had previously (*19*) characterized as carrying two nonallelic genes capable of controlling the fermentation of melibiose, although pedigree 2 proves that it carried only one *M* gene. Table 3 is the pedigree from our earlier paper. A

TABLE 3

*Pedigree 3:* Fermentation of melibiose by progeny of various crosses between *S. cerevisiae* (*m*) and *S. carlsbergensis* (*M*).

| | Ascus | Spores | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| *S. carlsbergensis* | C1 | M | | | |
| | C5 | M | M | M | M |
| | C6 | M | M | M | M |
| *S. cerevisiae* | Lk5 | m | m | m | m |
| Hybrid I | 1 | M | M | M | M |
| (Lk5B×C1A) | 2 | M | M | M | M |
| (*m*×*M*) | 3 | M | M | M | M |
| | 4 | M | M | m | M |
| | 5 | M | M | M | |
| | 6 | M | m | M | M |
| | 7 | M | m | M | |
| | 8 | M | m | M | m |
| Hybrid II | 1 | M | M | m | m |
| (Lk5B×Hybrid I-1A) | 2 | M | m | M | |
| (*m*×*M*) | 3 | M | m | M | m |
| | 4 | m | | M | m |
| Hybrid III | 1 | M | M | m | |
| (Hybrid I-1A×I-1D) | 2 | M | M | M | |
| (*M*×*M*) | 3 | M | M | M | M |
| | 4 | M | m | M | M |
| | 5 | M | M | M | |
| | 6 | | M | M | M |
| Hybrid IV | 1 | M | | m | m |
| (Lk5C×Hybrid I-1D) | 2 | M | m | | |
| (*m*×*M*) | 3 | m | m | M | |
| | 4 | M | m | M | m |
| | 5 | | | m | M |
| | 6 | | m | | M |
| | 7 | m | M | M | m |
| | 8 | M | M | m | m |
| Hybrid V | 1 | m | m | m | |
| (Lk5C×Hybrid IV-7D) | 2 | m | m | m | |
| (*m*×*m*) | | | | | |
| Hybrid VI | 1 | M | M | M | M |
| (C1A×Hybrid IV-7D) | 2 | m | M | M | |
| (*M*×*m*) | | | | | |

comparison of the two pedigrees (pedigree 3, C1A × Lk, and pedigree 2, C1A × *amg*) can be made by inspecting the analysis of Hybrids I and VI, Table 3, and of Hybrids I, II, and III, Table 2. In pedigree 3, one ascus in ten produced a 1:1 segregation of *M:m*, although two asci were incomplete. In pedigree 2, sixteen of eighteen asci gave 1:1 ratios of *M:m*. This high frequency of 1:1 segregation of *M:m* in pedigree 2 proves that C1A carries only a single *M* gene.

The excess of melibiose fermenters in pedigree 3

(C1A × Lk) shows that many of the recessive alleles were "masked" by the acquisition of the dominant phenotype. The earlier conclusion that C1A carried two genes controlling the fermentation of melibiose was incorrect, as is shown by the mating of C1A to *amg*.

Pedigrees 2 and 3 both describe cultures which were grown to maturity on glucose medium and then tested for their ability to ferment melibiose and galactose. (The importance of this fact will be shown later.) In pedigree 3, Hybrids II and IV
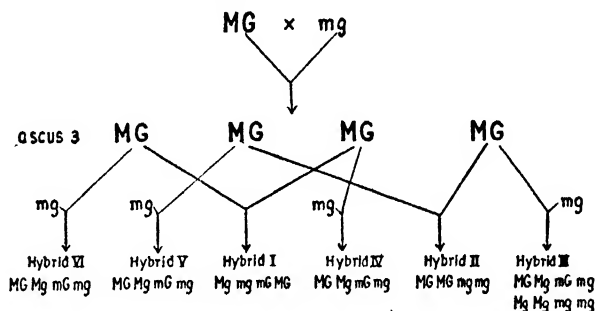


FIG. 2. A diagram showing the analysis of the exceptional ascus 3 (pedigree 2). Ascus 3 produced four spores, each with the dominant characteristics. Analyses of the intra-ascus crosses were made by mating spores A × C and B × D. Recessive alleles for both genes appeared in the progeny, thus proving that the phenotype (*MG*) of the spores in ascus 3 did not show the actual genotype. Each of the haploid cultures arising from the spores was backcrossed to the double recessive (*mg*). Analysis of backcross Hybrids IV, V, and VI revealed that in each case a 1:1 ratio for the fermentation or nonfermentation of both melibiose and galactose occurred, with one exception. This exception (Hybrid III) proved that this zygote was homozygous for the nonfermenting galactose allele and that spore D was genotypically *g* in spite of its ability to ferment galactose.

were made by backcrossing two of the haplophase cultures from an ascus from Hybrid I of the phenotype *MMMM* to the recessive *S. cerevisiae* parent. In each case a 1:1 ratio was obtained. At the time, this ratio was considered convincing evidence that a dominant-by-recessive mating had been made. However, 1:1 ratios may also be obtained by mating a "masked" recessive to a recessive (see Fig. 2). Since no further analysis was made of the earlier mating, the *M* parents used to make Hybrids II and IV may have been either "true" dominants or "masked" recessives.

Hybrid III was produced by crossing the two above-mentioned *M* parents with each other, and six asci were analyzed. Eighteen *M* progenies and two *m* progenies were obtained. At that time this mating was believed sufficient to prove that two dominant genes were involved, because it was assumed that the ability to ferment was adequate evidence of the possession of at least one dominant gene.

As a sequel to the paper containing the data shown in Table 3, Spiegelman, Lindegren, and Lindegren wrote a paper entitled "Maintenance and increase of a genetic character by a substrate-cytoplasmic interaction in the absence of the specific gene" (24). This paper described three experiments. (1) An $M \times m$ hybrid was made, and the first exposure of the haplophase segregants to melibiose occurred after the cultures were fully grown, just as in pedigree 3. The asci were all $MMmm$. (2) An $M \times m$ hybrid was produced and brought to sporulation in constant contact with melibiose. When exposure to melibiose was constantly maintained, the asci were all $MMMM$. (3) When the haplophase cultures from the second experiment were dissimilated in phosphate buffer in the absence of melibiose from seven to twenty days, two from each ascus permanently lost the ability to ferment melibiose.

On the basis of these experiments we concluded that some cells acquired the ability to ferment melibiose through cytoplasmic transfer from the dominant form. The $M$ parent used in these matings was I-1D, which was backcrossed to the recessive *S. cerevisiae* culture, Lk-5C. One-to-one ratios of $M$ to $m$ were obtained when this hybrid was produced in the absence of melibiose, whereas in the constant presence of melibiose the asci were all $MMMM$. With the help of Dr. Michael Doudoroff we have carried out several additional experiments on the effect of continued exposure to substrate. These experiments all yielded negative results, for there was no significant increase in the number of asci containing four fermenting spores in the presence of substrate, over the number of similar asci obtained from heterozygotes developed in the absence of substrate. For example, Hybrid I, pedigree 3, which was produced in the absence of melibiose, shows an excessively large number of $MMMM$ asci. Furthermore, some "masked" recessives, which have been carried in culture for more than a year in the absence of melibiose, are still able to ferment melibiose, suggesting that "masked" recessives produced in the absence of substrate may be indistinguishable from dominants under vegetative reproduction.

The fact that recessive progenies were obtained by mating I-1D with I-1A makes it impossible at the present time to state specifically whether I-1D carried the dominant gene, as was supposed at that time, or was a "masked" recessive. Therefore, although this experiment indicates that cytoplasmically acquired fermentative ability can be removed by dissimilation, it does not prove that "masked" recessives can *always* be purified by this simple procedure. As a matter of fact, some "masked" recessives, which have been carried in culture for more than a year in the absence of melibiose, are still able to ferment melibiose, suggesting that some "masked" recessives are extremely difficult to purify.

### Heredity among "Masked" Recessives

Regular Mendelian segregation of the genes controlling galactose and melibiose fermentation occurred in most of the asci listed in Table 2, but ascus 3, Hybrid I, was an exception. All four cultures from this ascus were capable of fermenting both sugars. The mating-type alleles segregated regularly in ascus 3, giving four cultures with the following characters:

| Spore | A | B | C | D |
|---|---|---|---|---|
| Character | $\alpha MG$ | $aMG$ | $aMG$ | $\alpha MG$ |

The analysis of the sister asci, in which "masking" of recessives did not obscure the Mendelian ratios, indicates that half of the spores in ascus 3 carried the recessive allele of $M$ and half carried the recessive allele of $G$. The genotypes in ascus 3 may have been either (a) $MG$, $MG$, $mg$, $mg$, (b) $MG$, $Mg$, $mG$, $mg$, or (c) $Mg$, $Mg$, $mG$, $mG$, although the phenotype of each culture was $MG$. Two of the spores with the $MG$ phenotype carried the dominant $M$, while two were "masked" recessive $m$. A similar obscuring of the 1:1 ratio of $G:g$ is also indicated.

*Intra-ascus crosses.* Corroboration of the view that some of the spores in ascus 3 were "masked" recessives, in spite of the fact that they showed the dominant phenotype, was obtained by making matings within the ascus (A $\times$ C and B $\times$ D, Table 4). Five asci from the A $\times$ C hybrid were dissected. A total of 10 spores was characterized as follows: $MG$, 5; $Mg$, 2; $mg$, 2; and $mG$, 1. The appearance of many nonfermenters shows that the hybrid was not homozygous for the dominant alleles.

In the hybrid B $\times$ D (D is genotypically $g$) 1:1 segregations occurred in the one complete ascus, and recessives appeared in an incomplete ascus. The hybrid was therefore not homozygous for either dominant.

The appearance of recessive alleles in the progenies of these intra-ascus crosses proves that the phenotype, $MG$, of the four spores in ascus 3 did not reveal the true genotypes.

In two of three matings, hybrids containing 3A produced only two viable spores per ascus. This may have arisen from several causes, but no attempt was made to analyze the situation further.

The appearance of nonfermenting cultures among the progenies of $MG \times MG$ matings shows that the supposed dominants are not capable of transmitting their newly acquired character through meiosis. The loss of the dominant character after mating a "masked" recessive with a dominant suggests that the "masked" recessive cannot usually retain its phenotype after meiosis with a dominant allele.

*Backcrosses.* Each of the four $MG$ cultures was backcrossed to the double recessive, $mg$. (Table 4, pedigree 4.) If we consider each allele separately, there were 4 backcrosses: (1) $M \times m$; (2) $G \times g$; (3) $m$ (masked) $\times m$; and (4) $g$ (masked) $\times g$. The first and second were simple dominant-recessive

hybrids, while the third and fourth hybrids were actually homozygous for the recessive genes, except that one of the recessive parents was "masked."

Irrespective of whether a dominant-recessive mating or a homozygous (recessive by masked re-

TABLE 4

*Pedigree 4:* Genetical analysis of the exceptional ascus 3, Hybrid I, pedigree 2, all of whose haplophase cultures were phenotypically *MG*. The analysis was obtained by making intra-ascus crosses, which revealed that recessive genes were present, and by backcrossing each one of the haplophase cultures to the double recessive, *mg*.

| Intra-Ascus Crosses | | | | | |
|---|---|---|---|---|---|
| Ascus | | Spores | | | |
| | | A | B | C | D |
| Hybrid I   *aMG/aMG* | 1 | *aMG* | *aMG* | | |
| Pedigree 2, Hybrid I, | 2 | *amg* | *aMg* | | |
| 3A×3C | 3 | *amg* | *aMg* | | |
| | 4 | *aMG* | *aMG* | | |
| | 5 | *amG* | *aMG* | | |
| Hybrid II   *aMG/aMG* | 1 | *aMG* | *amg* | *aMG* | *amg* |
| Pedigree 2, Hybrid I, | 2 | *amG* | *aMG* | *amg* | |
| 3B×3D | | | | | |

| Backcrosses | | | | | |
|---|---|---|---|---|---|
| Ascus | | Spores | | | |
| | | A | B | C | D |
| Hybrid III   *aMG/amg* | 1 | *amG* | *aMG* | *aMg* | *amg* |
| Pedigree 2, Hybrid I, | 2 | *aMG* | *aMg* | *amg* | *amG* |
| 3D×Pedigree 1, | 3 | *aMG* | *amg* | *aMG* | *amg* |
| Hybrid V, 7B | 4 | *amg* | *amG* | *aMG* | *aMg* |
| | 5 | *amg* | *aMg* | *aMg* | *amg* |
| Hybrid IV   *aMG/amg* | 1 | *amg* | *aMG* | *amg* | |
| Pedigree 2, Hybrid I, | 2 | *aMG* | *amg* | *amg* | *aMG* |
| 3C×Pedigree 1, | 3 | *amG* | *amg* | *aMg* | |
| Hybrid IV, 6D | 4 | *aMg* | *amg* | *amG* | |
| | 5 | | *amG* | *amG* | *aMg* |
| | 6 | *Mg* | *amG* | *aMG* | |
| | 7 | *aMG* | *amg* | *amG* | *aMg* |
| Hybrid V   *aMG/amg* | | | | | |
| Pedigree 2, Hybrid I, | 1 | *mG* | *amg* | *aMG* | |
| 3B×Pedigree 1, | 2 | *MG* | *mg* | *mG* | |
| Hybrid I, 2A | | | | | |
| Pedigree 2, Hybrid I, | | | | | |
| 3B×Pedigree 1, | 3 | *aMG* | *amG* | *amg* | |
| Hybrid V, 1A | 4 | *aMG* | *amg* | *aMg* | *amG* |
| Pedigree 2, Hybrid I, | | | | | |
| 3B×Pedigree 2, | 5 | *amg* | *amG* | *aMG* | *aMg* |
| Hybrid IV, 1B | 6 | *aMG* | *amg* | *mG* | |
| Hybrid VI   *aMG/amg* | | | | | |
| Pedigree 2, Hybrid I, | 1 | *amg* | *aMg* | *aMG* | *amG* |
| 3A×Pedigree 2, | 2 | *amG* | *amG* | *aMg* | |
| Hybrid IV, 1D | | | | | |
| Pedigree 2, Hybrid I, | 3 | *amg* | *amg* | | |
| 3A×Pedigree 2, | 4 | *amG* | *aMG* | | |
| Hybrid II, 2A | 5 | *aMg* | *aMG* | | |
| | 6 | *amG* | *aMG* | | |
| | 7 | *aMg* | *amg* | | |

cessive) mating was made, each ascus produced both dominant and recessive phenotypes. The recessive locus which had acquired the dominant character by contact with the dominant allele retained this phenotype after synapsis with the standard recessive allele.

The exceptional ascus 5, Hybrid III, Table 4, in which all four cultures were nonfermenters of galactose, reveals that the recessive allele is not always able to retain its cytogene, and points up the difference between dominant and recessive loci. It also indicates that culture 3D (ascus 3, Hybrid I, pedigree 2) is genotypically recessive for the *g* allele.

## NUCLEAR EQUIVALENTS IN SACCHAROMYCES

### *The Volutin Chromosomes*

In the diplophase the large central vacuole of the yeast cell contains twelve volutin chromosomes. Rafalko (22) has recently shown that threads can be found in the vacuole which take the Feulgen stain lightly. In the hemispherical centriole attached to the vacuole there are two strongly Feulgen-positive, rod-shaped bodies. In higher organisms, only the chromosomes are Feulgen-positive and desoxyribose nucleoprotein has been considered to be an essential constituent of the chromosome. However, some sections of the chromosomes take the Feulgen stain very densely (heterochromatin) and other sections take the stain lightly (euchromatin). Mazia and Jaeger (20) showed that in higher organisms the desoxyribose nucleic acids are secondarily attached to a basic chromosomal skeleton made up of histone or protamine. Stedman and Stedman (25) have found a protein (not histone or protamine) containing tryptophane to be an important chromosomal constituent, possibly a skeletal component of the chromosome.

There are two important facts that have not been sufficiently emphasized by biochemists interested in the chemical composition of the genes: (1) the chromatin (desoxyribose nucleoprotein) in higher plants and animals accumulates most abundantly in the regions of the chromosomes which contain few or no genes—namely, the heterochromatic regions of the chromosomes, and the Y chromosomes; (2) chromatin is not abundant in the resting nucleus when the genes are supposed to be functioning at a maximal rate of metabolic activity.

### *Volutin*[1]

Volutin is a cytological entity which is usually fixed with formaldehyde, although this fixation produces serious artifacts. It has never been chemically defined. It stains dark blue or red with methylene

[1] Wiame has recently identified volutin chemically as inorganic metaphosphate, and I have found that it is essential for chromosome division. Yeast chromosomes only divide when coated with metaphosphate, but they may contain little or no metaphosphate while in the resting condition.

blue and does not destain with 1% sulfuric acid. Babes described metachromatin in bacteria in 1889. Meyer named it volutin in 1904 and described its staining qualities. Guilliermond (8, 9) proposed changing the name volutin to metachromatin. He called the intravacuolar bodies in yeast (which I have identified as chromosomes) metachromatic granules, but he never described a well-fixed specimen. He called the cytoplasmic granules in yeast (which lose their stains when treated with dilute acid) basophilic granules.

Both Meyer and Guilliermond made extensive studies of the distribution of volutin in cells. Guilliermond (9) concluded that volutin is a reserve material like glycogen and fat, which is widely distributed, especially in the vacuoles of fungi and higher organisms. He considered the yeast vacuole to be homologous to the vacuoles of higher organisms and did not consider it part of the nuclear apparatus. In spite of the fact that he states that his tests for volutin were the same as those specified by Meyer, the legends of his drawings often indicate that the stain used was haemalum, which would stain the basophilic (cytoplasmic) granules equally well. This suggests the possibility that he may not have distinguished between volutin and cytoplasmic ribose nucleoprotein in higher organisms. He states specifically that metachromatin (volutin) passes from the vacuole of the yeast cell into the spores, but the details of meiosis have not been precisely worked out by anyone. Cells capable of sporulation contain numerous large cytoplasmic granules, which have impeded precise cytological study of meiosis in yeasts. It is very difficult to overstain and destain these cells in such a manner that one can distinguish critically between volutin and cytoplasmic granules.

I have devised a simple stain which detects volutin quite readily although it destroys the structure of the chromosomes. It is a mixture of acetic acid, formaldehyde, and toluidine blue or methylene blue. When properly adjusted it gives the same result with yeasts that Meyer's stain does, and with much greater regularity. Variations in formal-

dehyde make it necessary to adjust the stain from time to time. A standard formula is one part acetic acid, four parts saturated aqueous toluidine blue, and ten parts formalin. Cells are merely introduced into a drop of the stain and sealed under a cover glass. With this technique a culture of *Corynebacterium diphtheriae*, obtained from Dr. N. D. Duffett of the St. Louis Health Department, showed conspicuous and characteristic markings. This supports Guilliermond's view concerning the identity of metachromatin and volutin. This stain disintegrates or aggregates the yeast chromosomes, causing them to appear either as numerous small granules or one large globule, but it shows the characteristic substance in the vacuole (Fig. 3). Finer fixation techniques are required to avoid artifacts.

Wager and Peniston (26) incorrectly concluded that volutin occurs in the cytoplasm of the yeast cell, and engaged in a controversy with Guilliermond on this question. Caspersson and Brandt (6) have shown that ribose nucleoprotein is always present in the cytoplasm (either in granules or solution); but they found the vacuole to be optically empty with the ultraviolet microscope, indicating that it contained little nucleic acid. The combination of my findings and those of Caspersson and Brandt prove that volutin-Meyer (metachromatin-Babes, Guilliermond) is different from the cytoplasmic granules.

### The Cytoplasmic Granules; the Nucleolar Equivalents

In the cytoplasm of a resting (nonbudding) yeast cell one finds a variable number of refractive granules; some cells contain nearly fifty or more (Fig. 4). When the cell begins to divide these granules dissolve and the cytoplasm becomes optically homogeneous. These bodies in Saccharomyces were called basophilic granules by Guilliermond (8) because they stained with basic dyes. In an ultramicroscope study of Saccharomycodes (10) he called what are probably identical granules "lipoidal granules" because of their high refractivity. Wager and Peniston (26) and Caspersson and

LEGENDS FOR FIGURES 3 AND 4 (see opposite page)

FIG. 3. Results obtained using the volutin stain described in the text. In (a) the chromosomes stained but have been disintegrated, while in (b) they are balled up into a single globule.

FIG. 4. (a) and (b) Two different optical sections through a cluster of yeast cells which have just begun to bud. These cells were photographed with the Spencer phase-difference microscope. In the resting cells the cytoplasmic granules are very clear, with sharply delimited borders. Fewer and smaller cytoplasmic granules appear in the budding cells, showing that the granules become dispersed in the cytoplasm of an activated cell.

With the Spencer phase-difference lens the cytoplasmic granules are visible either stained or unstained and it has been possible to corroborate the findings of Caspersson and Brandt regarding their appearance in resting cells and their disappearance in active cells. This lens has given us a marked advantage over other workers, such as Wager and Peniston and Guilliermond, who used ordinary light microscopes, and whose work was done before Caspersson and Brandt had worked out the cyclical appearance and disappearance of the granules. Since many stains dissolve or obscure the granules, differences of opinion concerning their position or presence are understandable. They might easily be confused with volutin if large numbers are present, because the volutin stain, as devised by Meyer, requires destaining and this is always erratic through the yeast cell wall.
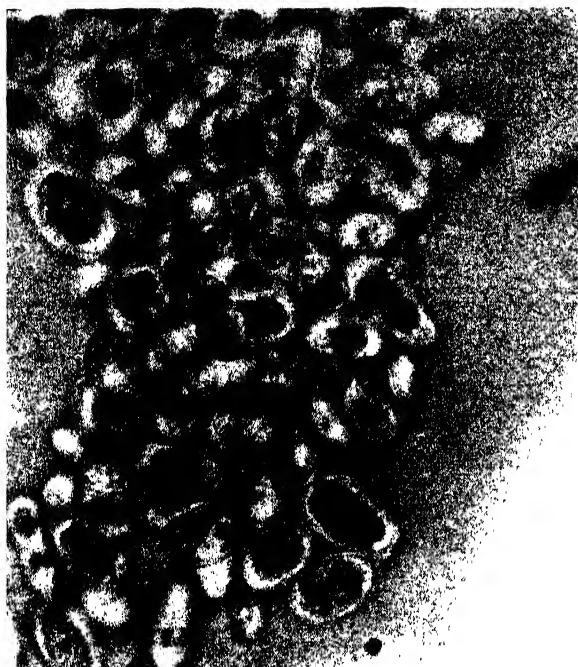
a

b

FIG. 3 (see opposite page for legend).
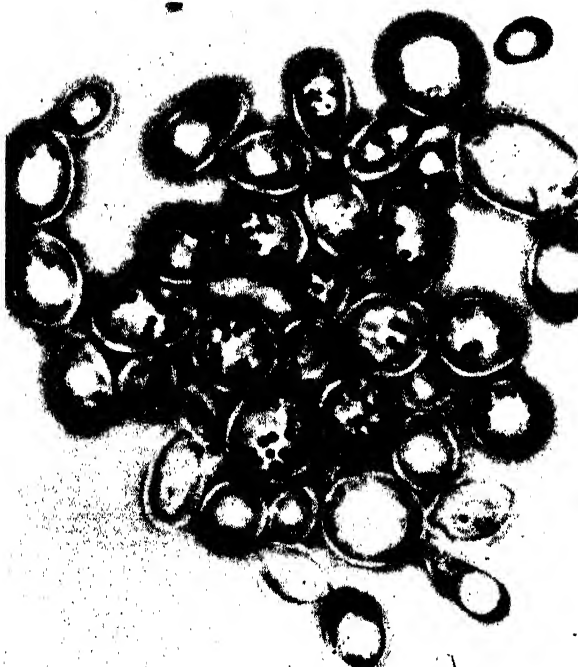


a
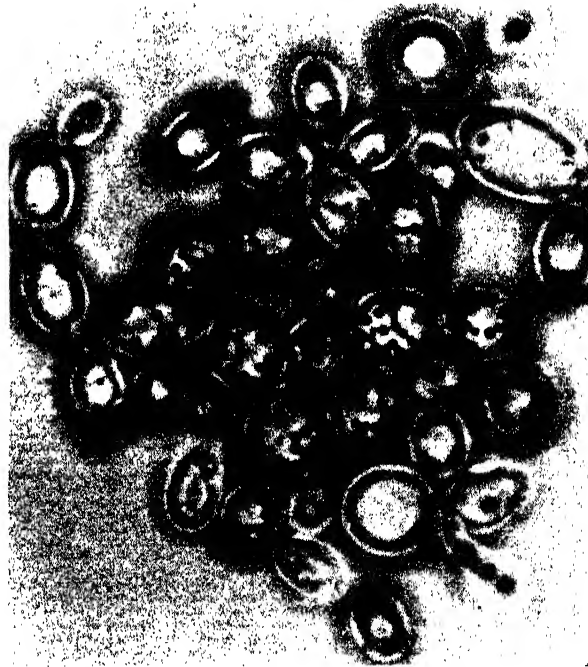
b

FIG. 4 (see opposite page for legend).
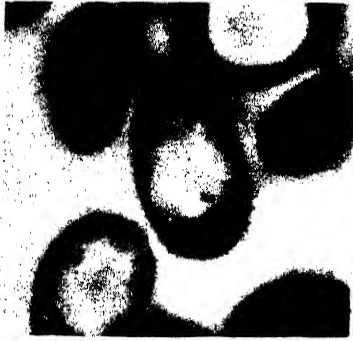
a

b

c

d
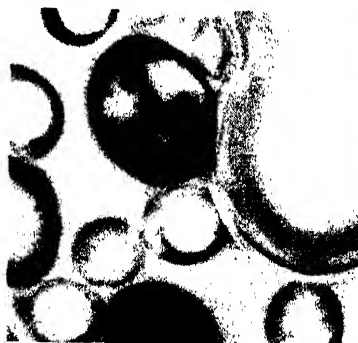
e

f
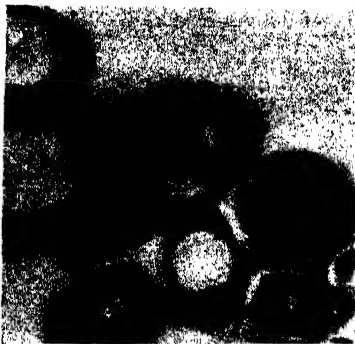
g

h

i
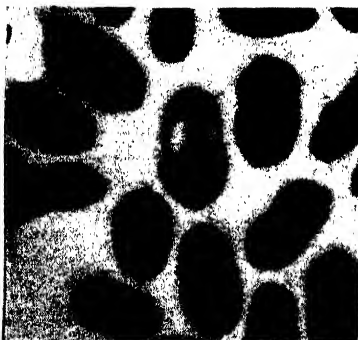
j

k

l

FIG. 6 (see opposite page for legend).
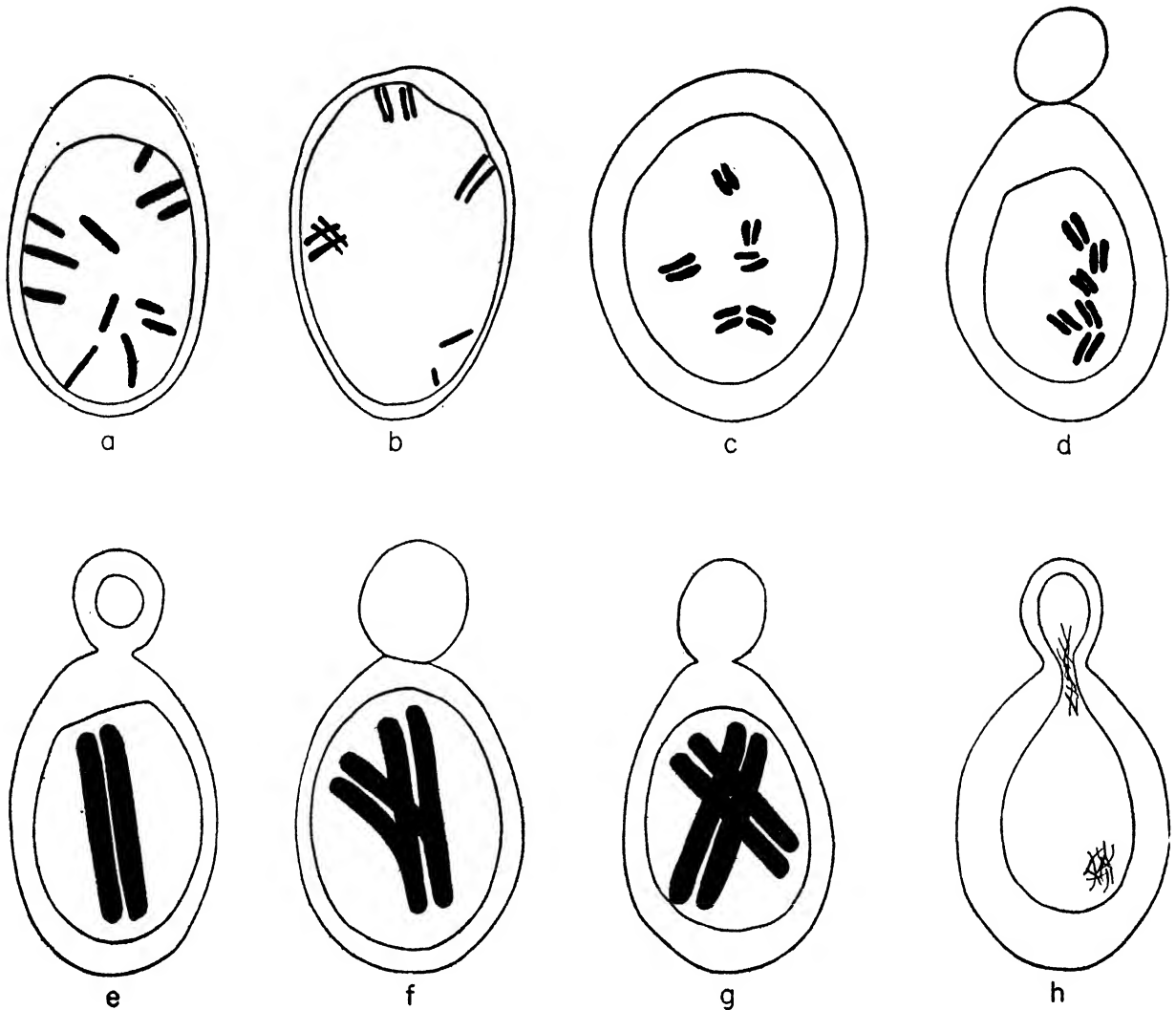
FIG. 5. Mitosis in the yeast cell. (a) Twelve chromosomes attached to the wall of the nuclear vacuole. (b) The chromosomes pairing up, still attached to the wall of the vacuole. (c) Six pairs of chromosomes free in the nuclear sap. (d) Aggregation of the six pairs into a complex. (e) A fully formed nuclear complex. (f) Longitudinal split producing two complexes. (g) Two separated complexes following the longitudinal split. (h) Chromosomes from one complex passing into the bud vacuole.

LEGEND FOR FIGURE 6 (see opposite page)

FIG. 6. (a) and (b) Twelve chromosomes attached to the wall of the nuclear vacuole. (a) is high focus and (b) is low focus of the same cell. (c) Five pairs of chromosomes attached to the wall of the vacuole. (d) One focus of a cell containing six pairs of chromosomes; only five pairs are visible in this optical section. (e), (f), (g), (h) Stages in the aggregation of the chromosomes. (i), (j) Fully formed complex with longitudinal split. (k) Cell with one uncondensed chromosome vibrating in the vacuole. Condensation has begun, as indicated by the knob on the distal end. (l) First division of a resting cell, showing two residual cytoplasmic granules, one in the mother cell and one in the bud. These probably correspond to the "chromatin patch" of Wager and Peniston. All but (f) are photographed with a Spencer phase-difference lens.

Brandt (6) called them volutin granules. I (15) have pointed out that this is erroneous, since volutin does not occur in the cytoplasm.

Caspersson and Brandt showed by precise ultraviolet microscopy that the cytoplasmic granules contain ribose nucleoprotein. Henneberg (11) and I (13) have called them fat granules because they are highly refractive; they coalesce when the cell is heated, or dead, or treated with alcohol, and are conspicuous in cells whose fat content is high. They are probably fat or lipoidal mixtures with ribose nucleoproteins. Caspersson suggested that the cytoplasmic granules are the equivalents of both heterochromatin and the nucleolus, since heterochromatin is associated in higher forms with the production of ribose nucleoproteins. It seems more logical, however, to consider the Feulgen-positive centriolar bodies the equivalents of heterochromatin.

The cytoplasmic granules do not change in appearance when the cells stand in 5% $Na_2CO_3$, which is known to extract ribose nucleoproteins rather effectively. They disappear when the cell is treated with acetic acid, which would not be expected if they contained only ribose nucleoprotein, but is characteristic of lipoidal bodies. They coalesce when the cell is heated, or treated with alcohol, or subjected to pressure, which is also consistent with the view that they contain lipoids or fats. They increase while fat storage occurs and are abundant in cells containing large amounts of fat. Precise information on their reaction to fat stains is difficult to obtain, because most fat stains enter the living cell only slowly or not at all. The surrounding cytoplasm contains considerable amounts of fat, so that fat stains quickly overstain the entire cell. In dead cells the structure is so changed that localization of fat may easily lead to false conclusions concerning its actual disposition in the living cell. Caspersson and Brandt pointed out that the cytoplasmic granules show an irregular boundary with the ultraviolet microscope in contrast to the nuclear vacuole. With the Spencer phase-difference lens that I have been using, the boundary between the cytoplasm and the cytoplasmic granule is very distinct (Fig. 4). These facts suggest that the cytoplasmic granules are lipoidal or fatty bodies containing ribose nucleoprotein. Caspersson and Brandt found optically empty fat vacuoles in the cytoplasm in certain old cells. These can easily be produced by heating or merely drying and fixing yeast containing a large number of cytoplasmic granules on a slide in the usual manner. They state that the optically empty bodies are probably the fat granules described by Henneberg, but it is clear from his figures that Henneberg called the smaller granules in the cytoplasm fat granules.

According to our view, the cytoplasmic granules are the equivalent of the nucleolus. There is no nucleolus apparent in the nuclear vacuole; the cytoplasmic granules break down when budding

begins, and the ribose nucleoprotein becomes dispersed in the cytoplasm. This is comparable to the process described by Caspersson in which ribose nucleoprotein passes from the nucleolus into the cytoplasm during the growth or activity of cells of higher organisms.

### The Feulgen-Positive Centriolar Bodies; the Heterochromatin Equivalents

The strongly Feulgen-positive character of the centriolar bodies suggests that they may be the equivalent in yeast of the heterochromatin in higher organisms. I (15) have already shown that the centriolar bodies cannot be the chromosomes because there are only two while genetical evidence requires a minimum of four chromosomes. There are two centriolar bodies in both haploid and diploid cells; they divide transversely instead of longitudinally at mitosis.

There seems to be a relation between the cytoplasmic granules and the centriole. The last cytoplasmic granules to disappear when budding begins are those at the base of the centriole (Fig. 6, l). Cytoplasmic granules often form a mantle surrounding the centriole (Fig. 4, a). These facts support the view that the ribose nucleoproteins are synthesized from the desoxyribose nucleoproteins found in the centriole. Caspersson and Schultz (7) and Schultz, Caspersson, and Aquilonius (23) found that ribose nucleoproteins in the nucleolus of Drosophila originated from the heterochromatin. They also found that synthesis of ribose nucleoprotein occurs in the cytoplasm at the boundary of the nuclear membrane.

### Mitosis in Saccharomyces

*Technique.* One of the first requirements in yeast cytology is that the cells should never be allowed to dry, for this produces abundant artifacts. Other sources of artifacts are strong killers and fixatives. Our present treatment is to kill the cells with dilute neutral red. The cell suspension is washed and a dilute stain, usually toluidine blue, is used. Saturated toluidine blue diluted from 1/40 to 1/800 is effective with some cultures. These preparations are evanescent, becoming rather rapidly overstained, but they are much better than those obtained by any other method. Other treatments such as aceto-orcein or IKI are useful to reveal different structures but they also should be used with wet mounts.

*Mitosis.* The twelve chromosomes in the diploid yeast cell are each attached by one end to the inner wall of the vacuole and wave around in the nuclear sap. They seem to be relatively rigid and are apparently rather evenly spaced about the inner wall of the vacuolar membrane. They are extraordinarily long in the living state, reaching across the entire vacuole and bending to conform to the inner aspect of the vacuolar membrane on the opposite side. When the twelve long chromo-

somes are waving about in the nuclear sap the vacuole is completely filled with vibrating chromosome threads. When killed, the chromosomes shrink down immediately to rather short rods, which still retain their equidistant disposition around the inner aspect of the nuclear membrane, but remain pointing inward (Figs. 5, a, and 6, a and b). Occasionally several of the chromosomes are polarized towards the centriole, but oftener they are equidistantly distributed around the entire inner surface. This disposition of the chromosomes means that their ends are in close proximity either to the ribose nucleoprotein granules or the ribose nucleoprotein dispersed in the cytoplasm, just as the chromosomes in higher organisms are in close proximity to the intranuclear nucleolus.

As mitosis begins, the homologous chromosomes approach each other but retain their attachment to the inner wall of the nuclear membrane, so that they are found in pairs with the distal ends waving around in the nuclear sap. Fixation causes them to shorten down to small cylindrical rods less than one-tenth their original length, but this also occurs in the natural course of events, producing six somatically paired chromosomes (Figs. 5, b, and 6, c). As soon as they have shortened down, they detach themselves from the nuclear membrane (Figs. 5, c, and 6, d). They vibrate in the vacuole in rapid Brownian movement, and finally begin to orient themselves into a single complex (Figs. 5, d, and 6, e, f, g, h). This process can be observed in a series of steps in which each one of the six pairs is at first distinctly identifiable but finally becomes merged into a single structure, which appears to consist only of two long, paired bands stretching across the entire diameter of the nuclear vacuole (Figs. 5, e, and 6, i and j). Occasionally these bands are twisted about each other. The chromosome complex is ribbon-shaped, about one-fifth as thick as it is wide. It has a pronounced longitudinal split and the appearance of two flat ribbons attached to each other by their edges. This flat characteristic becomes apparent when the six pairs are being arranged in the complex. The next step is the longitudinal splitting of the entire complex along the flat plane (Fig. 5, f). Partially split complexes, resembling Y-shaped figures, are often visible. It is also possible to find a pair of complexes in the cell, one lying across the other, each one extending the full distance of the vacuole (Fig. 5, g), often bending when they meet the inner surface. One of these complexes passes into the bud vacuole by slipping through the bud canal; and immediately afterwards, or during this passage (Fig. 5, h), the complex begins to break down to the twelve individual chromosomes.

## DISCUSSION

In one pedigree involving mating type and galactose fermentation, the ability to ferment galactose was inherited precisely as if it were controlled by a single Mendelian gene.

In a second pedigree, involving galactose fermentation, melibiose fermentation, and mating type, the factors controlling the fermentation of melibiose and galactose were transmitted in a Mendelian manner in most asci. In the occasional exceptions, three or four of the spores in an ascus were fermenters, indicating that some of the expected recessive (nonfermenting) progeny had acquired the ability to ferment. These "masked" recessives acquired the factor controlling fermentative ability (apparently at meiosis) and retained it in the absence of the respective substrates, galactose and melibiose. No simple scheme involving a cytoplasmic or a genetical mechanism could be invoked to explain this phenomenon. We had previously reported that a recessive could be transformed into a fermenter if the recessive spore was cut out of a heterozygous cytoplasm in the continued presence of the respective substrate. "Masked" recessives, in which the character was induced by this technique of direct transfer of cytoplasm, could be unmasked by dissimilation in the absence of the respective substrate. We recently made several attempts to duplicate this experiment, but were not able to produce more "masked" recessives by continued exposure to substrate than were found in the untreated controls. This does not necessarily invalidate our first experiment, but indicates, at least, that the conditions which control the phenomenon have not yet been completely worked out.

### Characteristics of "Masked" Recessives

"Masked" recessives that acquire their dominant characters in the absence of substrate are possibly different (either in kind or degree) from those produced by continued exposure of a heterozygote to substrate. In vegetative reproduction they are indistinguishable from the dominant; they retain the ability to adapt to substrate like the dominant, and in the dominant this ability apparently depends on the presence of a specific gene. Recessives that are masked by continued exposure to substrate during meiosis and subsequently unmasked by dissimilation may carry a factor controlling adaptation in the cytoplasm; while recessives that acquire the dominant character at meiosis may perpetuate the ability by some chromosomal mechanism. If recessives acquire the dominant character at meiosis in the absence of substrate and carry it on the chromosomes, they may obtain it by direct transfer from dominant chromogene to recessive chromogene. Many of our "masked" recessives are apparently capable of transmitting the dominant character indefinitely at mitosis (except for occasional mutations); but they transmit it irregularly at meiosis, depending upon whether they are mated to a "true" recessive or a "true" dominant.

In a preliminary paper I concluded that matings involving "masked" recessives yielded regular Mendelian ratios. More data indicate that these ratios may not be sufficiently regular to warrant this affirmation, and more study of this point will be required before final conclusions can be drawn.

When stable "masked" recessives are mated to "true" dominants, many recessives are recovered in the progeny; i.e., a "masked" recessive by a "true" dominant (fermenter × fermenter) yields numerous nonfermenters.

The mating of a stable "masked" recessive to a "true" recessive produces about half recessive and half "masked" recessive progeny; in this case the "masked" recessive retains the dominant phenotype after meiosis. The "masked" recessives *usually* retain the dominant character when mated to "true" recessives and *usually* lose it when mated to dominants. Therefore, a dominant gene retains its ability after synapsis with a "masked" recessive while the latter usually loses its newly acquired character; however, the "masked" recessive retains its ability after synapsis with a "true" recessive gene.

The fact that synapsis of dominant and "masked" recessive causes the latter to lose its synthetic capacity may be interpreted as due to removal of some substance from the recessive to the dominant allele. This would imply a greater affinity of the dominant chromogene either for a mechanism for synthesizing the cytogene or for the cytogene itself.

### Do Cytogenes Return to the Chromogene?

The irregular heredity demonstrated by yeasts was originally explained (*16*) by the cytogene theory. This theory stated (1) that the gene was a duality consisting of chromogene and cytogene, (2) that the cytogene was a substance stored at the chromogene whose synthesis occurred only in the cytoplasm, (3) that the dominant chromogene was merely a storage organelle, and (4) that the supply of cytogenes at the dominant chromogene was maintained by specific *return* of cytogenes *from* the cytoplasm *to* the dominant chromogene.

It is possible that the supply of cytogenes at the dominant chromogene is not maintained solely by the return of a specific cytogene *from* the cytoplasm *to* the chromogene, if more recent data revealing a relatively high frequency of 3:1 ratios resulting from backcrossing a "masked" recessive to a "true" recessive are confirmed. Three-to-one ratios would indicate that only a small number of cytogene particles was involved, because if recessives were masked by the return of cytogenes *from* the cytoplasm, and if a large number of particles were involved, both recessive alleles of equal strength in a heterozygous ascus would usually become transformed into "masked" recessives. On the other hand, if only a very small number of cytogenes were present, normal dominant-by-recessive crosses

would not regularly produce Mendelian ratios; because if the dominant chromogene depended only on *returned* cytogenes for its character it might frequently fail to show it, for the cytogenes might all go to one or the other of the dominant chromogenes. Therefore, the *return* of the cytogene *from* the cytoplasm *to* the chromogene is probably not the sole mechanism by which a dominant allele retains its ability to perpetuate its characteristic phenotype. In view of these fundamental contradictions—(a) that a small number would not give 3:1 ratios, and (b) that a large number would be necessary to insure the maintenance of the dominant gene's phenotype—we may conclude that the chromogene does not depend entirely upon cytoplasmic synthesis for its supply of cytogenes, but that synthesis of cytogenes occurs at the chromogene.

### Nonspecific Effects Producing Non-Mendelian Ratios

We have several indications that some nonspecific influence is an important factor in the irregular genetical behavior of yeasts. For example, we have developed several pedigrees recently in which the alleles controlling the fermentation of three sugars (galactose, melibiose, and maltose) are segregated in Mendelian ratios with a high degree of regularity. This is strong evidence for their basic genetical control. However, we have other closely related pedigrees, developed under apparently similar conditions, in which all three alleles simultaneously may exhibit very irregular, or non-Mendelian, inheritance. Of two asci from the same cross, one may show regular Mendelian behavior of all three genes (2:2 ratios) while the other shows irregular Mendelian behavior for all three genes (4:0, fermenter to nonfermenter). The fact that Mendelian inheritance may characterize all three alleles in one pedigree while predominantly non-Mendelian heredity may characterize the same three alleles in nearly all the asci in another closely related pedigree, together with the fact that usually all genes in one ascus are affected while usually all genes in another are not, indicates not an effect which is specific for each individual gene but rather some environmental effect acting upon the asci at the time of meiosis, which, depending upon conditions, influences a larger or smaller number of asci. It may be a local environmental effect which is very difficult to bring under precise control. I have already indicated that simply adding substrate is apparently not sufficient, although our earlier experiments indicated that it is an important factor. There is a similar situation in Neurospora, which we have studied in considerable detail. The ascospores in Neurospora are usually lemon-shaped, with two pores, one at each end. In some pedigrees an occasional ascus is found containing eight round spores with a single pore

each, although most of the asci ($>$ 99%) contain eight lemon-shaped spores (*18*). Mating a round single-pored by a round single-pored clone does not produce an increase in the number of round single-pored spores over that obtained from the standard two-pored by two-pored mating. In fact, the frequency of single-pored ascospores is apparently the same in single- by single-, single- by two-pored, and two-pored by two-pored matings. The outstanding characteristic of these exceptional asci is that every spore in the ascus is affected. It is an "all or none" effect. Either all spores in one ascus are normal or all are exceptional. This is clearly some cytoplasmic effect produced by a local and, at present, unpredictable environmental effect which is not transmitted to the progeny. Cytological analysis has indicated that it involves the timing of the spore-delimiting mechanism. Some similar local influence may produce the "masked" recessives which develop in the absence of substrate.

### Inhibitory Genes or Incompatible Cytoplasm

The possibility that the irregular non-Mendelian heredity may be under the control of a large number of genes has been considered. An attempt was made to formulate a genetical mechanism involving inhibitory genes, such as those suggested by Werner Braun, or compatible and incompatible cytoplasm (presumably under the control of genic action), as proposed by Spiegelman; but no simple formula could be worked out. For example, two haploid cultures showing the dominant fermenter phenotype (therefore presumably without inhibitors, or completely compatible) can be mated to produce a high percentage of recessive-nonfermenter, presumably inhibited progeny. Conversely, two parents derived from pedigrees showing highly regular Mendelian inheritance (in which the cytoplasm might be considered incompatible, or in which inhibitors are present) will produce a high percentage of recessives with the dominant (fermenter) phenotype. This is not expected if both parents are either inhibited or incompatible. A genetical scheme would require two or more linked genes, which produced reverse effects in recombinations. Schemes involving two pairs of alleles were examined. It was found that inhibitory or enhancing genes would be required to produce three-to-one ratios, thus making the genic formulation rather complex.

### Synthesis at the Chromogene

The view that the chromogene depends solely upon the cytoplasm for the cytogenes, which *return to* the chromogene *from* the cytoplasm after cytoplasmic synthesis, has not been supported by the data. Therefore cytogenes are probably synthesized at the chromogene. Multiple-factor inheritance does not offer any simple explanation of irregular non-Mendelian inheritance in yeasts. The

dominant chromogene seems to have greater affinity for either the synthesizing mechanism or the cytogenes themselves than the recessive chromogene, and exchanges between the alleles at meiosis in an irregular way might explain most of the data. At present it appears that the mechanism responsible for the irregular genetical behavior may be based on these premises: (1) the dominant chromogene can retain its synthetic capacity or its cytogenes more effectively than the recessive; (2) some mechanism or substance may be transferred irregularly from chromogene to chromogene at meiosis, often transforming regular Mendelian heredity into highly irregular heredity; (3) the dominant phenotype is not maintained exclusively by transfer of cytogenes *from* the cytoplasm *to* the chromogene; therefore, the cytogenes are probably synthesized at the dominant chromogene.

### Genic and Nongenic Self-Duplication

In spite of numerous exceptions, there are sufficient clear-cut pedigrees to establish the fact that the fermentations of the sugars galactose, melibiose, and maltose, are under the control of three separate, independently inherited genes. It has also been shown, however, equally unequivocally, that these fermentations are adaptive. That is, they occur only if the specific substrate is present; previous to this no measurable amount of the enzyme is present in the cytoplasm. Furthermore, the fermentative activity of the cell increases during the adaptation period by an interaction with substrate which is apparently independent of synthesis at the chromogene. These facts establish the concept of genic control or initiation of a substrate-cytoplasmic reaction which, once initiated, progresses independently to a maximal level. The presence of galactose permits the increase in amount of galactozymase in the cell up to this maximal level. Therefore, some self-duplicating entity multiplies in the cytoplasm, in the presence of substrate. (This is the substance which Spiegelman has called the plasmagene.) However, no entity can duplicate itself in a vacuum, and this self-duplicating entity is not distinguishable, in its ability to reproduce itself, from numerous other apparently self-duplicating entities in the cell like the mitochondria, the plasma membrane, the nuclear membrane (in ascomycetes), the centriole, etc. The principal question is whether or not it is different from the gene, which we have come to consider the basic, living, particulate substance. The gene is a self-perpetuating substance which differs from all others, as Muller has pointed out, in being able not only to duplicate itself but also to mutate and to continue to perpetuate the mutated form after mutation, in place of the original form. It will be difficult to determine whether the substance in the cytoplasm responsible for its fermentative capacity has the ability to mutate. We know, however, by many

other genetical experiments, that most apparently self-duplicating, cytoplasmic (nongenic) entities are altered in character by genic mutations and presumably only reflect changes in the gene without themselves having any power to change their characteristics by mutation. Therefore, the mutated gene produces a mutated cytogene, which acts to alter the specific character, or the specificity, of the phenotype. Sonneborn and Preer have shown that the kappa substance in Paramecium is able to duplicate itself in the presence of the killer gene but unable to duplicate itself in its absence. It follows, therefore, that there is an intermediary substance given off by the killer gene which is an essential nutrient for the kappa substance. This intermediary substance is either the cytogene itself or something which is brought into being in the cytoplasm by an interaction between the cytogene and the kappa substance. The kappa substance may then be maintained like a virus, which depends on a nutrient produced by a single gene.

### Possible Relation between Cytogene and Nucleoproteins

The facts concerning the genetics of sugar fermentation in yeasts can now be examined in relation to the cytological mechanism. The nuclear vacuole contains an abundance of volutin together with a small amount of desoxyribose nucleoprotein (22).

Desoxyribose nucleoprotein is found in the centriole, but the rest of the cytoplasm is invariably negative for this substance. In contrast, ribose nucleoprotein is found throughout the cytoplasm during rapid growth and fermentation, but is aggregated into granules, coated with lipoids, when the cell is dormant. The cytoplasmic ribose nucleoproteins probably originate from the desoxyribose nucleoproteins of the centriole. This view is based on the extensive work of Caspersson and Brandt on yeasts and the work of Schultz, Caspersson, and Aquilonius (23) on the general problem of the interrelationships of desoxyribose nucleoprotein and ribose nucleoprotein. The centriole is not surrounded by a membrane, for its outline in aceto-orcein or in untreated cells is diffuse. The sharp outline it assumes when stained with iodine is due to coagulation.

On the basis of this information it may be profitable to speculate on the relationship of the cytogene to the chromosomes and to the cytoplasmic ribose nucleoprotein. The following working hypothesis is proposed. In the nuclear vacuole the cytogene is attached to the chromogene. Each chromogene is differentiated from its neighbor in such a way as to insure the attachment of the cytogene. The cytogene can pass freely through the nuclear membrane of yeast, which is impermeable to both the cytoplasmic ribose nucleoprotein and the nuclear desoxyribose nucleoprotein. When the cytogene is present in the cytoplasm it is attached to the ribose nucleoprotein. The ribose nucleoprotein carrying the cytogene in the cytoplasm is the precursor of the enzyme, which gives the enzyme its specificity in the presence of the specific substrate. The cytogene may be of relatively small molecular weight and confer specificity to a particle of much larger weight. This is reasonable, since we know that the synthesis of vitamins, presumably by enzymes of high molecular weight, can be blocked by vitamin homologues of relatively low molecular weight. If a small-moleculed vitamin homologue can specifically block the synthesis of a vitamin, it seems reasonable that a small-moleculed substance can confer specificity to the enzyme controlling the synthesis. The cytogene-ribose nucleoprotein complex (the *plasmagene* of Spiegelman) attains the ability to duplicate itself in the cytoplasm *only when substrate is present*. The cytogene may be similarly attached as a prosthetic group to the desoxyribose nucleoprotein in the nucleus. However, in the nucleus it is maintained and multiplies *in the absence of substrate*.

According to this view, specific desoxyribose nucleoproteins, the chromogenes, have the ability to enable specific cytogenes to multiply on the chromosome in the absence of substrate; whereas in the cytoplasm the cytogenes multiply in the presence of substrate attached as prosthetic groups to ribose nucleoproteins.

### SUMMARY

Tetrad analysis of Saccharomyces hybrids revealed occasional high frequencies of non-Mendelian segregations. Many recessive alleles apparently acquired the dominant character at meiosis. Genetical analysis of these "masked" recessives revealed that they usually lost the character when mated to "true" dominants, but usually retained it when mated to "true" recessives. Cytological study of the yeast cell disclosed that the nuclear vacuole contains twelve chromosomes. The cytoplasm contains ribose nucleoprotein granules coated with lipoidal material when the cell is dormant. In the growing cell, the ribose nucleoprotein becomes dispersed in the cytoplasm and the cytoplasmic granules disappear. The following hypothesis is suggested. The cytogene multiplies in the nucleus as a prosthetic group on the desoxyribose nucleoproteins, independently of substrate; whereas, in the cytoplasm, the cytogene multiplies as a prosthetic group on the ribose nucleoprotein in the presence of substrate.

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## DISCUSSION

EPHRUSSI: I should like Dr. Lindegren to make a statement concerning his present views on the problem of mating types and heterothallism in yeasts. I think this is important at a moment when many people are going into yeast work, and I feel sure that a discussion of this problem would inevitably have arisen had Dr. Winge been able to attend these meetings.

In his early papers on yeasts Dr. Lindegren claimed, on the basis of experiments with a strain of *S. cerevisiae*, that yeasts are sharply differentiated into two mating types, *a* and *α*. The table which summarized his results showed a certain number of exceptions, or "illegitimate" diploids. It was stated, however, that the latter either never form asci, or (if they do so) form asci only with abnormal numbers (two or three) of ascospores; and that if, exceptionally, asci with four spores are produced, the ascospores never germinate. These results were extrapolated to yeasts in general, and similar statements can be found in Lindegren's more recent publications.

Dr. Winge, on the contrary, has claimed since 1935 that yeast cells are sexually undifferentiated.

In the course of recent work on the genetics of yeast I and my collaborators have found that in these organisms the situation with respect to sex is highly flexible. We have isolated several haploid strains from *S. cerevisiae* Baker's yeast II from the collection of the Pasteur Institute, and in agreement with Dr. Lindegren have found that in some of these there is a sharp differentiation into + and − lines, within which "illegitimate" diploidization apparently never occurs. However, other haploid strains have been isolated from the same original culture which, after a period of haplophase growth, frequently undergo "illegitimate" diploidization by cell fusion, a process which does not prevent them from forming four-spored asci nor the ascospores from germinating. Although this is at variance with Lindegren's generalizations, such four-spored "illegitimate" asci have been used with success in some of Lindegren's own and Spiegelman's experiments.

It may be mentioned also that a pronounced change in mating behavior is often associated with mutational changes.

While this very flexible situation does not prevent the detection of sex differentiation in some lines, in others no symptoms of heterothallism have so far been found. Thus, in agreement with Winge's results, we find that in monosporic cultures of *S. cerevisiae* var. *ellipsoideus* (strain Pouilly II) diploidization by fusion of haploid vegetative cells is the rule.

These results indicate that no generalization based on observations of a few strains, varieties, or species is at present justified.

Lastly, I should like to add that the high frequency of the so-called illegitimate diploidizations calls for great caution in the interpretation of the results of crosses which involve the fusion of vegetative cells.

LINDEGREN: I am aware of the exceptions to a rigid system of heterothallism that Dr. Ephrussi has pointed out, as well as many others which I have observed. In fact, I have published several papers, some of which have dealt exclusively with exceptions of this type. A mycologist conceives of heterothallism as a system in which numerous exceptions occur. Brunswick in 1924 invented the term *"Durchbrechungskopulation"* to describe exceptions to the standard scheme for sex reaction in Coprinus, and recognized that many fungi produce fruiting bodies directly from the haplophase. Hanna in 1925 showed that, while one may find regular segregation of mating types in the fruiting bodies isolated from one dung heap, testing for mating types with the spores from a neighboring dung heap may give complete fertility. Vandendries in 1929 made an extended study of exceptions to the standard sexual behavior in a number of hymenomycetes, and originated the term "illegitimate" for irregularities in copulation.

It is now a well-accepted fact that Neurospora is a heterothallic fungus, but sterility which obscures the inheritance of the mating-type alleles is frequently encountered in this species. We once described two peculiar Neurospora cultures, which behaved like + and — (i.e., they could be mated to each other) but each of which also produced perithecia when mated to either of our standard + or — mating types. These cultures were found to be self-sterile bisexual heterocaryons, and the haplophase cultures, segregating from hybrids with them, showed an exceptionally high degree of sterility. Moreau and Moruzi showed that similar, heterocaryotic cultures could be induced to produce perithecia simply by transmission of hormones through the agar.

In spite of the evidence of many sterility factors in Neurospora, we have never observed transformation of a + into a — mating type, or vice versa. The zygote is always heterozygous for the +/— gene pair. Andes, however, working with *Glomerella cingulata*, has shown that mating type in this fungus in quite unstable and transformations occur with high frequency, and also that the zygote may be homozygous or heterozygous. Nevertheless, this is clearly a heterothallic fungus. In older yeast cultures irregularities of this type are occasionally found, as well as transformations into promiscuous sexual mutants.

These exceptions are all embraced in the mycologist's conventional concept of heterothallism. In yeasts we were the first to show that a single pair of genes controls mating-type specificity, but also showed that exceptional individuals could be found that were completely promiscuous, mating freely with both types; and we found in addition that mating-type specificity was easily lost simply by continued transferring of the culture, although it was retained when the cultures were lyophilized. One of our most interesting exceptions involved yeasts which were capable of producing ascospores directly in the round haploid cells without even passing through the stage of illegitimate copulation. I have just recently learned that we were not the first to observe that the spores obtained from single ascospore cultures were generally inviable. This was first reported by Šatava in Prague in 1934; incidentally, this contradicts the view that European yeasts may differ markedly from American.

I was, of course, familiar with the work of Winge, who showed that a single ascospore culture could produce four viable spores. We also found this to occur in some strains, but, in the majority of cases, an ascus that produces four haploid cultures usually produces two of each mating type. Descriptions of heterothallism in yeasts were always qualified, except when the information was presented to a nonbiological, or lay, audience, and in the present paper I have assumed that all of you are familiar with the work we have already published on the subject.

We have always been aware of the possibility that *MMMM* asci from *M* × *m* hybrids, or that *MG MG MG MG* asci from the *MG* × *mg* hybrids, may be illegitimate offspring of the *M* or the *MG* parents. We constantly guard against it by critically testing both parents for their ability to sporulate when grown alone. None of the haploid parents in the pedigrees presented above was able to sporulate.

Critical proof that a heterozygous *Me/me* hybrid may produce *Me Me Me Me* asci was obtained in the following experiment. We analyzed a heterozygote of the following type: *a g Me ma* × *a G me Ma* (*Ma* = maltose). In 12 asci from this hybrid, the *a/a*, *G/g*, *Me/me*, and *Ma/ma* genes segregated regularly, proving that each was a hybrid. In 4 exceptional asci, the *a/a*, *G/g*, and *Ma/ma* genes segregated regularly, but these 4 asci were *Me Me Me Me*. These exceptional asci could not possibly have arisen illegitimately from the *a g Me ma* parent, since it carried neither the *a*, the *G*, nor the *Ma* genes.

SONNEBORN: Lindegren's conception of the cyto-

gene must be regarded merely as a working hypothesis and one which, because of its radical nature, calls for relentless criticism and the most unobjectionable experiments until it is securely established or discarded. Lindegren's chief evidence for the hypothesis is derived essentially from the analysis of one exceptional ascus: one with 4 *MG* spores obtained and grown in the absence of both melibiose and galactose (see his Table 4).

One needs the strongest possible experimental evidence that 2 of the 4 spores in this ascus differ from the other 2 in the way postulated by Lindegren. The experiments attempting to show this fail in my opinion to provide this needed evidence. What is required is to cross the haplophase cultures from these 4 spores, separately and individually, both to known *mg* cultures and to known *MG* cultures. The latter, which should yield some *m* and *g* in two, and only two, of the four crosses, is most critical and has not been reported at all. The former (cross to recessives) has been reported. The results are interpreted by Lindegren to mean that some of the apparently dominant parents were not true dominants, but "masked" recessives. This may be true, but to be convincing the analysis needs to be carried out on a sufficiently large scale to demonstrate conclusively that such results are obtainable from two, and only two, of the four haplophase cultures under investigation. In the experiment the required results were obtained for only one of the four haplophase cultures and on a scale not adequate to demonstrate that they could not have occurred equally well for all four. Until the full analysis, as here called for, is provided, the experimental foundation on which the cytogene hypothesis is built must be considered inadequate.

As Lindegren rightly maintains, the various exceptions to simple Mendelian inheritance, as shown in his Tables 2 and 3, are the fundamental observations requiring explanation. To be sure, they can formally be interpreted in accordance with Lindegren's hypothesis, but formal accord, it seems to me, is insufficient ground on which to base so important a hypothesis, particularly since the hypothesis is plastic enough to be readily reconcilable with any such exceptions. An alternative hypothesis, based on multiple factors, which seems to me particularly worth full exploration, is dismissed by the Lindegrens because it "does not offer any simple explanation." To many geneticists a multiple-factor explanation would be preferable even if it were not simple. In the end, however, the only satisfactory conclusions will be those based on a full experimental analysis, which, it is to be hoped, will in time be provided.

LINDEGREN: Sonneborn insists that it is necessary to present the "strongest possible experimental evidence" that the "masked" recessives be distinguishable from the standard dominants. Actually, many of the "masked" recessives are as indistinguishable from dominants as transformed Type III pneumococci derived from Type I roughs are from the standard Type III. The important thing is that a transformation has occurred, and whether or not the "masked" recessive can be distinguished from the standard dominant has no bearing at all on the question. In many cases the transformation is nearly complete and the backcross to the dominant which Sonneborn required to produce recessives may not always do so.

I pointed out that the multiple-factor hypothesis which Dr. Sonneborn prefers would be too elaborate to be justified even with the present data. Additional evidence has now conclusively excluded it from consideration.

# SPONTANEOUS BACTERIAL MUTATIONS TO RESISTANCE TO ANTIBACTERIAL AGENTS

## S. E. LURIA

Bacterial genetics differs from the genetics of higher organisms chiefly because of lack of information about the mechanism of orderly transmission of characters from generation to generation, owing to the absence of well-established processes of conjugation and to the infant state of bacterial cytology. Our only way of approach is the study of bacterial mutability, for which the field of bacterial variation offers a wide range of material. One of the first tasks we face is that of establishing how closely mutations in bacteria are comparable to the so-called gene mutations in higher organisms. Geneticists as well as bacteriologists have often thought that the genetic system of bacteria may be less orderly; in particular, that properties may be transmitted at fission by more or less random distribution of substances present in the protoplasm, without special mechanisms for equational division.

If a case could be made, however, for similarity of the processes of mutation in bacteria and in higher organisms—that is, for the existence of discrete, gene-like hereditary units in bacteria—then these organisms might prove to be invaluable material not only for the study of physiological genetics, but also for an attack on the problems of gene structure and mutability (4, 5).

The case would necessarily rest on analogies, some of which may be listed as follows: (1) permanence of the mutated character; (2) "spontaneous" occurrence, independent of specific environmental stimuli; (3) definite rate of occurrence; (4) independence of different mutations in the same organism; (5) inducibility by agents known to produce mutations in higher organisms; (6) reversibility, showing that the mutation is not necessarily a loss, caused by unequal division or some similar process; (7) physiological effects, furnishing proof that bacterial mutations can bring about the same type of metabolic changes known to be produced by gene mutations in higher organisms.

One difficulty of bacterial genetics is that of recognizing a given mutational step, since the same character may be affected by a number of different mutations, which we cannot attribute to different genetic loci by classical genetic tests. Hence the necessity of identifying each mutation by as many of its phenotypic effects as possible.

Another peculiarity of bacterial genetics is that we generally do not deal with the properties of single individuals, but only with those of large "clones" (colonies, single-cell cultures), each derived from one individual whose characters must be retraced from those of the clone. We must therefore take into account intraclonal variability. Cases of high mutation frequency are generally unsuitable for the study of the mutation process, since they are likely to be balanced by reverse mutations; our visible clones will then contain mutant and reverted types in genetic equilibrium. These considerations have been discussed in greater detail by Delbrück (8).

More convenient are cases with low mutation frequency, since these need not necessarily be balanced by either reversion or adverse selection. In these cases the mutants can be detected, however, only if we can select them out in a suitable environment. Mutants capable of growth in media "incomplete" for the wild type (22, 19) are in this category. Other such mutations are those producing increased resistance to some destructive agent.

We shall discuss in this paper the latter group of mutations, on the basis of work on bacterial resistance to bacteriophages, antibiotics, drugs, and radiation (18, 11, 1, 2, 9, 24, 21).

## ORIGIN OF RESISTANT VARIANTS

The resistant variants are generally isolated as colonies which appear after the parent strain has been plated in the presence of a destructive agent. The question then arises: were the cells that gave these colonies resistant before the plating, or have they acquired hereditary resistance as a response to the action of the antibacterial agent? Unappealing as the second alternative may be to classical genetic thought, the question is by no means idle. Considerable evidence has been interpreted as supporting the idea that environmental conditions, in particular nutritional or inhibitory stimuli, may alter the metabolic machinery of the bacterial cell, not only temporarily, but in a permanent hereditary way (14, 13). It may be conceivable that a small fraction of the cells exposed to an antibacterial agent respond to its action with a specific change in properties, and that this change is somehow transmitted to the offspring.

Mutational origin of the resistant variants, independent of the action of the destructive agent, can only be proved either by direct isolation of resistant substrains before the treatment (7), or by establishing the presence within sensitive cultures of "clones" of resistant bacteria, each clone stemming from a mutation that occurred during the growth of the cultures prior to the test for resistance (18). If we define mutation rate as the probability that a

bacterium will mutate in a given interval of time, the frequency of mutations will be proportional to the number of bacteria present. Each mutation gives origin to a "clone" of mutants; the earlier the mutation occurs, the larger the clone derived from it will be at the moment of the test. If the mutants grow at the same rate as the normal bacteria, clones of a certain "size" will be twice as frequent as those of double that size. The expectation should be, therefore, that, of the mutants present in a culture at any one time, equal numbers stem from mutations that occurred in each of the previous generations. This situation, however, is never realized in actual cases. In any actual culture, the likelihood

same culture. A detailed analysis of these considerations has been given by Luria and Delbrück (*18*).

These expectations can best be tested in the case of resistance to bacteriophages. The phage-resistant variants are easily detected and counted in a mixture with sensitive cells; they neither grow the phage nor adsorb it, and their resistance is a permanent new character of the strain.

As an example, Table 1 (from *18*) shows the results of the enumeration of bacteria resistant to phage T1 in platings from similar cultures of *Escherichia coli,* strain B. The presence and type of fluctuations in the frequency of mutants provide strong confirmation of the mutational origin of re-

TABLE 1. DISTRIBUTION OF THE NUMBERS OF BACTERIA RESISTANT TO PHAGE T1 IN SERIES OF SIMILAR CULTURES OF SENSITIVE BACTERIA

| Experiment No. | 22 | | 23 | |
|---|---|---|---|---|
| Number of cultures | 100 | | 87 | |
| Volume of cultures, cc. | .2* | | .2* | |
| Volume of samples, cc. | .05 | | .2 | |
| | Resistant Bacteria | Number of Cultures | Resistant Bacteria | Number of Cultures |
| | 0 | 57 | 0 | 29 |
| | 1 | 20 | 1 | 17 |
| | 2 | 5 | 2 | 4 |
| | 3 | 2 | 3 | 3 |
| | 4 | 3 | 4 | 3 |
| | 5 | 1 | 5 | 2 |
| | 6–10 | 7 | 6–10 | 5 |
| | 11–20 | 2 | 11–20 | 6 |
| | 21–50 | 2 | 21–50 | 7 |
| | 51–100 | 0 | 51–100 | 5 |
| | 101–200 | 0 | 101–200 | 2 |
| | 201–500 | 0 | 201–500 | 4 |
| | 501–1000 | 1 | 501–1000 | 0 |
| Average per sample | 10.12 | | 28.6 | |
| Variance (corrected for sampling) | 6270 | | 6431 | |
| Average per culture | 40.48 | | 28.6 | |
| Bacteria per culture | $2.8 \times 10^8$ | | $2.4 \times 10^8$ | |

* Cultures in synthetic medium.

of occurrence of early mutations is small, because not enough individuals are present. When the population reaches a size where mutations begin to occur, the frequency of their occurrence will be subject to tremendous fluctuations owing to their small average number. In a large number of trials (series of similar cultures), therefore, we must expect to find corresponding large fluctuations of the frequency of mutants; the presence of these fluctuations is a direct consequence of the clonal distribution of the mutants. When the proportion of variants in a series of similar cultures shows such large fluctuations, we may interpret them as proof of a clonal distribution, hence of a mutational origin of the variants.

Such fluctuations among similar cultures would not be expected if resistance were actively produced by the antibacterial agent. The fluctuations should not be greater than those among samples from the

sistance. A refinement of this type of analysis would be afforded by a comparison of the actual distribution of the number of variant individuals with a distribution calculated from the assumptions of the mutation hypothesis. Mathematical difficulties have been encountered, however, in attempts to calculate the theoretical distribution.

It is important to notice in Table 1 (Exp. 23, in which the whole contents of the cultures were tested) the presence of large numbers of cultures containing one mutant only. This suggests that the spontaneous mutations may be phenotypically expressed without a lag longer than one cell generation.

The "fluctuation" test has been applied to a variety of cases—resistance of *E. coli* B to several phages (*11*) and to radiation (*24*); resistance of Staphylococcus 313 to penicillin (*9*), and to sulfathiazole (*21*)—with results of the same type, indicat-

ing that in all these cases resistance arises as the result of mutations occurring before the test. The data of Lewis (16) on *Escherichia coli mutabile* show that similar fluctuations between cultures were present in the numbers of lactose-fermenting cells. Large fluctuations in the time of appearance of fermentation variants were also present in experiments of Kristensen (15). The mutational origin of bacterial variants is well supported by all these observations.

The type of test described above shows only that the variants were already a genetically modified type before the test. It is possible that in some cases the specific environment, besides selecting out the mutants, may also act by rendering phenotypical a new character otherwise masked by some cytoplasmic effect. In a sense, this may be considered to be true of mutations affecting the production of adaptive enzymes. In the case of mutations to resistance to antibacterial agents, similar instances might be difficult to detect.

## MUTATION RATES

In work on bacteriophage resistance, Luria and Delbrück (18) have chosen as a definition of the mutation rate the probability of mutation per bacterium per physiological time unit (generation). This definition is satisfactory as long as it can be proved that mutations occur only during growth of the bacteria, and that their frequency is proportional to the physiological rather than to the astronomical time. These conditions appeared to be fulfilled in our experiments on phage resistance (mutation rate independent of generation time in different media, no increase in mutants after maximum growth).

Several methods have been proposed for estimating the mutation rate. Since mutations occur independently of each other, when there are only a few they will be distributed among different cultures in a Poisson distribution, with the probability of any given number of mutations occurring in one culture depending only on the average number of mutations per culture.

The average number of mutations per culture, and hence the mutation rate, can be calculated from the number of cultures having no mutants and from the number of bacteria per culture. If in a series of C similar cultures, each containing N bacteria, a fraction $C_0/C$ proves to contain no mutant (the whole contents of the cultures must obviously be tested), the mutation rate, a, can be obtained:

$$(1) \quad a = -\frac{\ln 2 \times \ln (C_0/C)}{N}$$

This method, although it requires careful planning of the experiments, has the advantage of not introducing the actual numbers of mutants and thus avoiding complications due to possible selection.

Another method that allows direct measurement

of mutation rates without making use of the numbers of mutants consists in applying the antibacterial agent (phage) to bacteria growing on solid medium without disturbing the formation of microcolonies (10). One colony appears for each *mutation* that has occurred. The difficulty here lies in precise estimation of the number of bacteria present at the moment of the test.

Mutation rate can also be calculated from the actual number of mutants, estimating from this the number of clones present. One must take into account that in cultures of limited size mutations are likely to occur only during the last part of their growth. If, for example, the mutation rate is $10^{-7}$, mutations are not likely to occur, in actual cultures, until the population reaches a size of the order of $10^7$ cells. Introducing the necessary corrections, the mutation rate can be estimated from the average number, r, of mutants from C similar cultures:

$$(2) \quad r = \frac{aN}{\ln 2} \ln \frac{aNC}{\ln 2}$$

A diagram for the use of this formula has been given by Luria and Delbrück (18). Mutation rates calculated by formula (2) are likely to be inaccurate because of the fluctuations in frequency of occurrence of the early mutations; in particular, they will be too high whenever one or more of the unlikely early mutations happens to occur. Important corrections should be introduced if the growth rates of normal and mutant types are different. Altogether, our present methods of estimating mutation rates are far from satisfactory.

Some values of mutation rates calculated with formulas (1) and (2) are given as examples in Table 2. These values are subject to the further limitation that all mutations giving the same phenotype for the character investigated are lumped together, and that in some cases it is likely that not all mutants present in a sample are detected. The orders of magnitude, however, are probably correct.

Even for the same wild-type strains, mutation rates for different mutations to resistance vary over a wide range. That they are generally very small is to be expected; in searching for stable resistance to destructive agents, we particularly select for rare mutations, since if stable mutants occurred very frequently they would generally have displaced the parent type, unless checked by strong adverse selection. Much higher mutation rates may be found for mutants that also show high rates of reversion.

## INDEPENDENT MUTATIONS

It has already been mentioned that the same bacterium can undergo different mutations. This is clearly seen in the case of resistance to bacteriophages. Working with *Escherichia coli* B and bacteriophages T1-T7, one finds a series of bacterial mutations, each producing resistance to one, two,

or more phages. Indicating a mutant from B that is resistant to phage n by the symbol B/n, we have for instance the mutants: B/1; B/1, 5; B/6; B/3, 4, 7. From each of these mutants one can obtain other mutants with additional resistance to other phages: B/1/6; B/6/3, 4, 7, etc.

The successive mutations are clearly independent. A strain that has undergone a certain mutation will generally give the same mutations to resistance to other phages as the parent wild-type strain. Within the limits of precision of the available methods of measurement, the rates for the same mutation in the parent strain and in any of its mutants appear

could decide by what mechanism resistance to unrelated phages is produced in one mutational step. The answer to this question might be of the greatest importance, also, in relation to the mechanism of bacteriophage action.

A possible interpretation has been suggested by E. H. Anderson's work on the nutritional requirements of some phage-resistant mutants (*1*, *2*). Anderson found that a number of B/1 mutant strains from *Escherichia coli* B differed from the wild type also in being unable to grow in a minimal synthetic medium. The metabolic disturbance was identified as inability to synthesize *l*-trypto-

TABLE 2. MUTATION RATES

| Organism | Mutation | Reference | Mutation Rate per Bacterium per Generation | |
|---|---|---|---|---|
| | | | Formula (1) | Formula (2) |
| *Escherichia coli* B | Resistance to phage T1 | (*18*) | $3 \times 10^{-9}$ | $1.7 \times 10^{-8}$ |
| | Resistance to phage T1 | (*11*) | $7 \times 10^{-9}$ | $2.7 \times 10^{-8}$ |
| | Resistance to phage T3 | (*11*) | $5 \times 10^{-8}$ | $1.2 \times 10^{-7}$ |
| | Resistance to phage T6 | (*11*) | $5 \times 10^{-8}$ | $\sim2 \times 10^{-7}$ |
| | Resistance to radiation | (*24*) | | $\sim10^{-6}$ |
| *Staphylococcus aureus* 313 | Resistance to penicillin, one-step | (*9*) | | $\sim10^{-7}$ |
| | Resistance to sulfathiazole, one-step | (*21*) | $\sim10^{-9}$ | |

to be equal. For example, the mutations B → B/1, and B/6 → B/6/1 occur with the same frequency (*11*).

The same holds true for other types of mutation as well. The mutant B/r resistant to radiation (*24*) has the same pattern of mutability to phage resistance as the wild-type B.

These results provide evidence of the independence of different mutations in bacteria, and agree with the hypothesis of the existence of discrete, mutable, gene-like units.

## MULTIPLE EFFECTS OF MUTATIONS

In cases of resistance to drugs and antibiotics, the resistance is often very specific; cross-resistance is limited to related substances. In the case of phage resistance, however, multiple resistance to several phages may appear as one mutational step. The phages to which resistance can be produced by one mutation are often unrelated as judged by their size, structure, serological specificity, and growth characteristics (*8*). It is easy to prove, on the basis of the mutation rates, that the multiple resistance does not result from chance occurrence of two or more independent mutations in the same bacterial line. Moreover, resistance to the same group of phages can be reached in one or two mutational steps. It is clear, therefore, that tests of resistance to a limited number of phages are not sufficient to characterize the genotypes of a group of bacterial mutants.

It would be an important step forward if we

phane, which therefore became a required nutrilite for these strains. Moreover, these strains exhibited another disturbance in their nitrogen metabolism, reflected by their inability to utilize inorganic nitrogen in the absence of at least one of a series of amino acids.

Additional inability to synthesize the amino acid proline was found by Anderson in a strain B/1/3, 4, 7. Other phage-resistant strains that fail to grow in a minimal medium have also been encountered.

Anderson (*1*) interpreted his findings as indicating the existence of common steps in the chains of reactions leading to the synthesis of an essential metabolite and of substances needed for phage sensitivity, the mutation blocking one of the enzymatic reactions. As an extension of this interpretation, it was suggested that multiple resistance to various phages acquired in one mutational step results from the blocking of a reaction common to the syntheses of substances needed for growth or adsorption of the phages. Common steps could occur in the chains of reactions leading to sensitivity to unrelated phages. Different mutations producing resistance to more phages would probably produce blocks at different levels of the series of reactions.

Since bacteria resistant to a certain phage are often sensitive to some mutants from that phage, the "blocks" in the chains of synthetic reactions are likely to alter, rather than suppress completely, the synthesis of substances necessary for phage sensitivity.

It was thought (*17*) that the mutational changes might simply involve slight alterations in the configuration of the bacterial receptors for phage adsorption, which were supposed to react with the phage by simple coming together of complementary surface structures. Mutational alteration of the surface receptors could be brought about either by a primary change in the receptor itself or by an alteration in the structure of a gene-like center acting as template for the receptor, without involvement of any long reaction chain. The association of specific metabolic deficiencies with phage resistance seems to contradict this idea. Moreover, the finding of T. F. Anderson (*3*) that certain amino

phage-resistant mutant of the usual type. Unfortunately, the relation between the two types of resistance was not thoroughly analyzed. The indications were, however, that phage resistance in the acridine-resistant strain was caused by a change in an acridine-sensitive mechanism involved in phage growth. Interestingly enough, both antiphage action of the acridines and phage resistance of the acridine-resistant strain could be antagonized by the addition of ribose nucleic acid.

The idea that the usual type of phage resistance is due to a block in a chain of synthetic reactions would be convincingly proved if it were possible to restore sensitivity to a resistant mutant by supply-

TABLE 3. ONE-STEP MUTANTS

| Phenotype | T1 | T5 | T2 | T6 | T4 | T3 | T7 | T7h | T7h' * | Tryptophane Requirement | Generation Time, Minutes, in Broth at 37° C. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | S | S | S | S | S | S | S | S | S | — | 19–20 |
| Frequent mutants | | | | | | | | | | | |
| a | R | S | S | S | S | S | S | S | S | + | 19–20 |
| b | R | R | S | S | S | S | S | S | S | — | 19–20 |
| c | S | S | S | R | S | S | S | S | S | — | 19–20 |
| d | S | S | S | S | R | R | S | S | S | — | 25–26 |
| e | S | S | S | S | R | R | R | S | S | — | 25–26 |
| f | S | S | (S) | (S) | R | R | R | R | S | — | 29–30 |
| Complex mutants | | | | | | | | | | | |
| g | R | S | (S) | (S) | R | R | R | R | S | + | 29–30 |
| h | R | R | (S) | (S) | R | R | R | R | S | — | 28–29 |
| i | S | S | R | R | R | R | R | S | S | — | 28–29 |
| l | R | S | R | R | R | R | R | S | R | + | 40 |

* The braces indicate serological relationship between phages.

acids may act as necessary cofactors for phage adsorption indicates that the latter process is likely to involve some rather complex enzymatic activity of the phage particle on the bacterial surface.

We found recently that some of the B/3, 4, 7 mutants, besides not adsorbing phages T3, T4, and T7, also show a partial inability to grow phages T2 and T6. They adsorb these phages like normal wild-type bacteria, but 80-90% of the infected bacteria fail to liberate any phage at all. Similar observations were also made by Hershey (personal communication). This phenomenon, which needs further investigation, indicates a relation between adsorption capacity and ability to grow phages, and supports the idea that both phenomena involve chains of reactions variously linked.

Additional evidence for relating phage resistance to specific changes in enzymatic reactions is offered by recent results of Fitzgerald and his collaborators (*12*). It was found that some acridines exerted a specific inhibition on phage growth in the bacterial cell, and that a variant resistant to one of the acridines was almost as resistant to phage as a

ing its medium with the intermediate metabolite whose synthesis is supposedly blocked, and whose absence is assumed to be responsible for the phage resistance. Such cases have not yet been found.

Some additional information on multiple resistance has been derived from a category of rare mutants with unusual combinations of resistance to various phages (*11*). We have recently isolated a number of these "complex" mutants, and attempted a further characterization of their phenotypes, comparing them with those of the more common mutants. This was done by taking into account sensitivity to phages T1-T7 and to some of their host-range mutants (active on some of the resistant bacterial mutants), nutritional requirements, and growth rates, which will be considered later. The complex mutants were isolated by plating large numbers of sensitive bacteria in the presence of suitable mixtures of phages. Most of them occur with mutation rates of the order of $10^{-10}$. The results of some of these tests are summarized in Table 3.

We see that certain basic groupings of characters

appear in the phenotypes of both simple and complex mutants: resistance to phages T1 and T5; to phages T3, T4, and T7; resistance to phage T1 and tryptophane requirement. All of the complex mutants incapable of growing in minimal medium were found to have the same tryptophane requirement as the B/1 mutants, although some of them grow more poorly in the presence of the same amount of the amino acid.

The coupling of resistance to phage T2 and to phage T6, as present in the complex mutants, is not shown by any of our simple mutants. This seems due to the fact that all the frequent mutants isolated from our B strain in the presence of phage T2 prove sensitive to this phage after growth in phage-free media, owing to some as yet unclear mechanism of
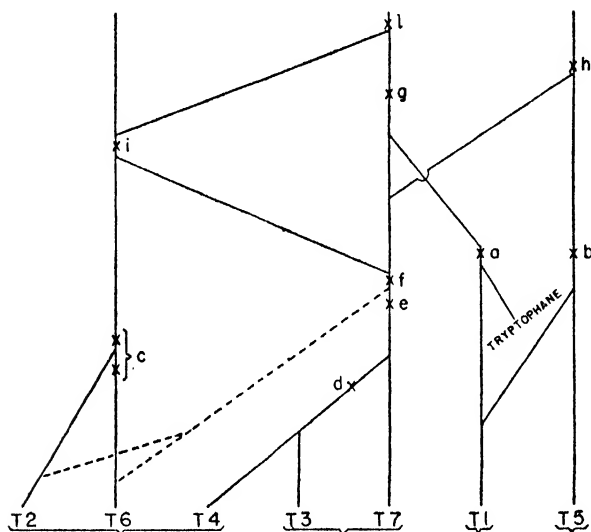


Fig. 1. Chains of synthetic reactions needed to account for the coupling of resistance in the mutants of Table 3. The symbol X in each case indicates the block corresponding to the mutant phenotype indicated by the adjacent letter. The broken lines indicate the coupling of resistance to T3, T4, T7 with failure to grow T2 and T6.

partial reversion to sensitivity. Thus most of the B/2, 6 strains show an apparent B/6 phentoype (phenotype c, Table 3).

The groups of characters shown by the "simple" mutants appear in the complex mutants, associated in a number of ways but not in all possible ways. For example, resistance to phages T3, T4, T7 was found associated either with resistance to T1 and T5 (phenotype h) or with resistance to T1 and tryptophane requirement (phenotype g), or with resistance to T2 and T6 (phenotype i). Resistance to phages T1 and T5 was not found associated with tryptophane requirement.

Some of the complex phenotypes are clearly a superposition of two of the simpler ones: phenotype g is like phenotypes a + f; phenotype h, like b + f. Other complex mutants differ from the sum

of two simple ones in some character, particularly in sensitivity to various mutants from T7.

It may be possible to account for these various combinations of characters, according to Anderson's scheme, on the basis of blocks or changes in common steps of different chains of synthetic reactions, assuming that each phenotype corresponds to a different synthetic block; the situation, as shown in the diagram (Fig. 1), becomes quite complicated. One must assume that there are a number of different reactions common to the chains leading to sensitivity to certain groups of phages, and that some of these reactions are shared by different chains of reactions leading to sensitivity to other phages. Further complications are revealed by differences in sensitivity to various mutants of the T7h group, indicating either a greater multiplicity of pathways, or different alterations of the same reaction. Each of the numerous reactions assumed in order to explain multiple resistance to such a small number of phages must be capable of being altered by a nonlethal mutation. We should also assume that the rare frequency of occurrence of all the more complex mutations is purely coincidental.

One way out of some of these difficulties would be to suppose that some or all of the very complex mutations are actually combinations of two or more simple mutations occurring simultaneously. Such simultaneous occurrence might be brought about by some type of mass rearrangement of the genetic material of the bacterial cell, possibly comparable to that responsible for chromosomal rearrangements in higher organisms.

Altogether, we feel that the problem of multiple effects of a bacterial mutation cannot yet be interpreted satisfactorily by any single hypothesis. Recognition of a larger number of complex mutants, their characterization by as many traits as possible, study of the physiology of the changes involved, and work on reverse mutations should help clarify the still obscure situation. Cases of simultaneous acquisition of several growth requirements may offer good material for this study.

### QUANTITATIVE RESISTANCE

In studying the acquisition of resistance to antibiotics (penicillin, 9) and drugs (sulfathiazole, 21), it has been found that resistance to increasing concentrations of an antibacterial agent is built up stepwise by accumulation of successive mutations, each contributing further resistance, until strains are obtained that for all practical purposes can be considered as completely resistant. Mutants isolated by the selective action of a certain concentration of an agent are often resistant to much higher concentrations. Supposed correspondence of the resistance level with the concentration used in the "training" to an antibacterial agent has been considered (13) as one proof of the active induction of resistance. The actual lack of such correspondence

is corroborating evidence of its spontaneous, mutational origin. The successive mutations to resistance might represent either similar changes in a series of gene-like units, or successive changes in the same unit, or independent genetic changes each affecting one of the metabolic processes on which the antibacterial agent may act.

A study of sulfonamide resistance in Staphylococcus (21) indicates that the last alternative is probably correct. A number of different mutations are involved in acquisition of this resistance, most of the recognized ones occurring at comparable rates (between $10^{-9}$ and $10^{-10}$). These mutations can be differentiated first of all by the degree of resistance produced, expressed by the drug concentration withstood by the mutant. Even more important is the fact that different mutants with increased sulfonamide resistance differ in other correlated characters, particularly in the production of an extracellular sulfonamide antagonist apparently identical with p-aminobenzoic acid. Some of the resistant mutants release during growth up to 30 times more p.a.b. than the parent strain, while others do not produce any increased amount of it. The same mutation affecting p.a.b. production may occur at the same rate either in the parent strain or in mutants with already increased resistance but without increased p.a.b. production.

The simplest explanation is that sulfonamides act as inhibitors of a number of metabolic reactions (whether all involving p.a.b. or not is immaterial for our problem). Successive mutations to resistance produce changes in different enzymatic mechanisms, the residual level of sensitivity being determined by the most sensitive mechanism left.

In the case of penicillin resistance (9), the occurrence of mutations producing different degrees of resistance indicates a similar situation. That penicillin affects a number of different enzyme systems of the bacterial cell is indirectly indicated by data of Treffers (23) on growth inhibition brought about by associations of penicillin with each of a number of metabolic poisons in noninhibitory amounts.

The acquisition of resistance to drugs and antibiotics by mutation in bacteria seems, therefore, to fall in line with the idea of genetic determination of single enzyme reactions by individual genes. It is interesting to note that, in the case of resistance to radiation in *E. coli* B, Witkin (24) found only one mutational step increasing resistance. All attempts to select mutants having higher resistance failed. It is likely that only one of the synthetic mechanisms responsible for sensitivity to radiation can be modified by a nonlethal genetic change.

The increased production of p.a.b. by sulfonamide-resistant mutants of Staphylococcus exemplifies a metabolic change increasing the extracellular production of a metabolite. P.a.b. is not a required metabolite, and is normally produced by the organ-

ism. It is possible, on the one hand, that p.a.b. accumulates because of a block in a sulfonamide-sensitive reaction that utilizes p.a.b. Provided an alternative metabolic pathway for growth were available, such a block would be expected to produce sulfonamide resistance. It is possible, on the other hand, that an actual increase in p.a.b. synthesis is caused by the mutation. That positive biochemical mutations may be responsible for resistance to antibacterial drugs seems indicated by the increased formation of pantothenate by *C. diphtheriae* in the course of its becoming pantoyltaurine-resistant and independent of pantothenate for growth (20).

## GROWTH OF THE RESISTANT MUTANTS

The resistant mutants often show definite differences in growth characteristics from the sensitive wild-type parents, which can be used as additional criteria for identification of a given mutational step.

In the case of phage-resistant mutants, some mutations are correlated with definite changes in growth rate (generation time). Some of the data for the growth of one-step mutants in nutrient broth at 37° C. are included in Table 3. The growth rates of the mutants range from the same value as for wild-type to values more than twice as great. These differences again indicate that the mutations produce profound changes in the metabolic processes of the bacterial cell.

It was found that when successive mutations are accumulated in the same bacterium the growth rate is always determined by the mutation that by itself gives the slowest growth.

We can see in Table 3 that those complex mutants that appear to be the superposition of two simple mutant types also have the same generation time as the slower simple mutant type included. This agrees with the suggestion that they actually originate by combined mutations. Others of the most complex mutants have a much slower division time, probably indicating a more profound alteration of their synthetic abilities.

The effects on growth of mutations to resistance are not limited to changes of generation time in the logarithmic phase. All phases of growth may be affected. Among mutants resistant to phages or to penicillin, we found some having longer or shorter lag phases, others with higher or lower maximum titers than the wild-type strain.

Of a large number of mutants whose growth was measured under comparable conditions, a majority grew less well than their wild-type parents. Only a few seemed to grow as well or better. This seems to agree with the general principle that most mutations arising in well-established wild-types produce less favorable phenotypes.

Complications may arise, however, when one attempts to predict the growth of parent and mutant strains in mixtures on the basis of their growth in isolated cultures. Production of diffusible growth

inhibitors or stimulants, or differential rates of utilization of some nutrilite, may greatly alter the picture. For instance, we found that the viable counts of a penicillin-resistant mutant in mixed culture with the parent strain began to fall long before the end of the growth phase for separate cultures of either strain. Other instances of interactions of this type have also been encountered. In different phases of the life of a bacterial culture either the parent type or its mutants may be favored, and only detailed study of individual cases can clarify the most complex situations. The indications are, however, that many cases of bacterial variation, including some of the most complex types of so-called life cycles, can be explained in terms of the simplest hypothesis—mutation followed by selection within the resulting mixtures of phenotypes (6).

## SUMMARY

Bacterial mutations to resistance have provided favorable material for the quantitative study of bacterial mutability. The mutations discussed in this paper present suggestive analogies with gene mutations in higher organisms, although the similarity of the genetic systems involved can only be considered, for the time being, as a useful working hypothesis. The problems of determination of mutation rates, of identification of single mutation steps in strains with different genotypes, and of multiple effects of mutations have been discussed.

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## DISCUSSION

KIDD: I wonder if Dr. Luria has seen a resemblance between the complex races of phage-resistant bacteria with which he has been working and the strains of drug-fast trypanosomes which have interested parasitologists since the time of Ehrlich. While the genesis of the drug-fast strains of trypanosomes has not perhaps been particularly well studied as judged by the criteria of critical quantitative genetics, the fact has long been known that they represent more or less stable heritable variations in microorganisms. Furthermore, the work of Warrington Yorke and his collaborators and that of Jancsó and others has made it abundantly plain that the lethal effects of drugs on trypanosomes depend upon their fixation by the parasites: arsenical and acridine compounds, for example, are absorbed by strains of normal trypanosomes, which then succumb to their

effects; but the compounds are not absorbed by the drug-fast strains, which in this way resist their action. From a study of the phenomena of drug resistance, moreover, Frank Hawking has distinguished four different kinds of receptors in trypanosomes—those for arsenicals and acriflavine, for parafuchsin, for diamidine compounds, and for Bayer 205—and he has studied the relationships among them.

It seems to me that the parasitologists have managed to acquire a noteworthy comprehension of the phenomena with which they were confronted— the practical implications for chemotherapy included—and it may become manifest that the principles involved are much the same as those with which Dr. Luria is dealing.

SONNEBORN: The finding of a single mutant colony when an entire culture is plated out need not necessarily indicate that the mutation was brought to phenotypic expression at once in the absence of further cell reproduction. Suppose the mutation had occurred several cell generations earlier, with a lag of some cell generations between mutation and phenotypic expression and with variation among the progeny of the mutated cell in the number of cell generations preceding phenotypic expression. In Paramecium, for example, precisely such variation is observed. Under such conditions a single descendant cell might resist phage and the others be destroyed. It would then be difficult to distinguish this from cases of immediate phenotypic expression.

# SOME PROBLEMS CONNECTED WITH SPONTANEOUS BIOCHEMICAL MUTATIONS IN BACTERIA

## ANDRÉ LWOFF

This report will consider a limited number of spontaneous biochemical mutations recently studied by the author and his colleagues of the Pasteur Institute's department of bacterial physiology. These cases have been selected as a basis for the discussion of a number of important problems connected with this vast subject. They will be considered under two headings: (1) mutations involving a change in the metabolism of ternary compounds; and (2) mutations involving a change in the power to synthesize essential metabolites (growth-factor mutations).

### MUTATIONAL CHANGES IN THE METABOLISM OF TERNARY SUBSTANCE

#### *Anaphragmic Mutations* (see definition on p. 143)

*The dicarboxylic-acid mutation of Moraxella lwoffi.* The genus Moraxella (*21*), which belongs to the Bacteriacea, groups diplobacillary, nonmotile, noncapsulated, nonsporogenic, Gram-negative bacteria, generally parasites of the oculary mucosa. *Moraxella lwoffi* var. *brevis* (*2*) is able to grow in synthetic media with an ammonium salt as a nitrogen source. A great variety of organic molecules (though not one carbohydrate) may serve as the sole carbon source (*23*). With succinic acid, growth starts only after a negative period of from 3 to 20 days which varies according to the size of the inoculum.

From various experiments with liquid media, and platings on succinate-synthetic media (*24*), it appeared that this negative period corresponds to the selection of a mutant. The mutated form seems to be quite stable: mutated bacteria grown with acetate through numerous transfers maintain the ability to utilize succinic acid. The same phenomenon occurs with fumaric and *l*-malic acids.

We shall refer to the normal form as $N$ and to the mutants as $S^+$, $F^+$, and $M^+$ bacteria.

The simplest hypothesis appeared to be that $N$ bacteria are unable to attack succinic, fumaric, or malic acid and that $S^+$, $F^+$, and $M^+$ bacteria represented independent mutations. It was found, however, that all the mutated strains, whether selected on succinic, fumaric, or *l*-malic acid, were able to utilize any one of these three acids. The hypothesis of independent mutations of the three different specific enzymes (succinic-acid dehydrogenase, fumarase, malic-acid dehydrogenase) was,

then, quite out of the question. It was obvious that we were dealing with a single mutation. This mutation could be supposed to involve either:

(1) A change in a common precursor of the three specific enzymes.

(2) A modification of an enzyme involved in the transformation of a single substance which would be a common metabolic product of the three $C_4$ dicarboxylic acids. According to classical conceptions of the metabolism of dicarboxylic acids, the common metabolic product should be oxaloacetic acid (which will be referred to as o.a.a.).

(3) A change of an enzyme attacking $C_4$ dicarboxylic acids, possibly through phosphorylation.

As far as could be seen, the $S^+$ mutation is connected only with the $C_4$ dicarboxylic acids. The metabolism of glutaric and glutamic acid, not utilized by $N$ bacteria, is affected by two mutations, which are independent of each other as well as of the $S^+$ mutation. It is possible to obtain accumulation of these three mutations in the same bacterium (*3*).

A comparative study of the metabolism of $N$ and $S^+$ clones gave the following results (*25, 26*).

*Respiration.* (1) $N$ bacteria take up oxygen in the presence of acetate or lactate. The rate is greater in the presence of an ammonium salt. Controls without added substrate have a very small though measurable respiration. With succinate or malate, $O_2$ uptake is very slow and difficult to measure (Fig. 1, A). If heavy suspensions are used, however, the succinate respiration is easily estimated and it is seen that it decreases rapidly with time (Fig. 1, B). There is no measurable respiration in the presence of o.a.a.

$C_4$ dicarboxylic acids (succinic, fumaric, malic, and o.a.a.) do not inhibit the oxidation of acetate or lactate.

(2) $S^+$ bacteria respire and grow normally (in the respirometer) with lactate, acetate, succinate, fumarate, or malate as carbon sources. Respiration is also normal with o.a.a. No growth experiments were performed with o.a.a., since it is spontaneously decarboxylated to pyruvic acid, which is used by both $N$ and $S^+$ bacteria.

*Production of o.a.a.* The slow and decreasing oxidation of succinic, fumaric, and malic acids by $N$ bacteria gives rise to o.a.a., as shown by assays of the o.a.a. produced (Fig. 2). With malic acid the rate of production depends on the concentration of the substrate. (The hypothesis that a small part of

the o.a.a. produced could be destroyed will be discussed later.) The rate of its production is nevertheless very low, and agrees roughly with the rate of $O_2$ uptake.

Despite the fact that, as we shall see, o.a.a. is rapidly destroyed by $S^+$ bacteria, it can be detected in $S^+$ cultures. The speed of o.a.a. production at the expense of malic acid is about the same with $S^+$ and with $N$ bacteria (Fig. 3, A and $B_1$).

Oxygen consumption by $S^+$ bacteria in the presence of malate is much greater (over 10 times) than would be required for the production of the o.a.a. actually found. This favors the view that malate may be attacked otherwise than by dehydrogenation.

It is, of course, possible that the *estimated* o.a.a. merely represents the *difference* between o.a.a. *production* and o.a.a. *destruction*. This hypothesis,
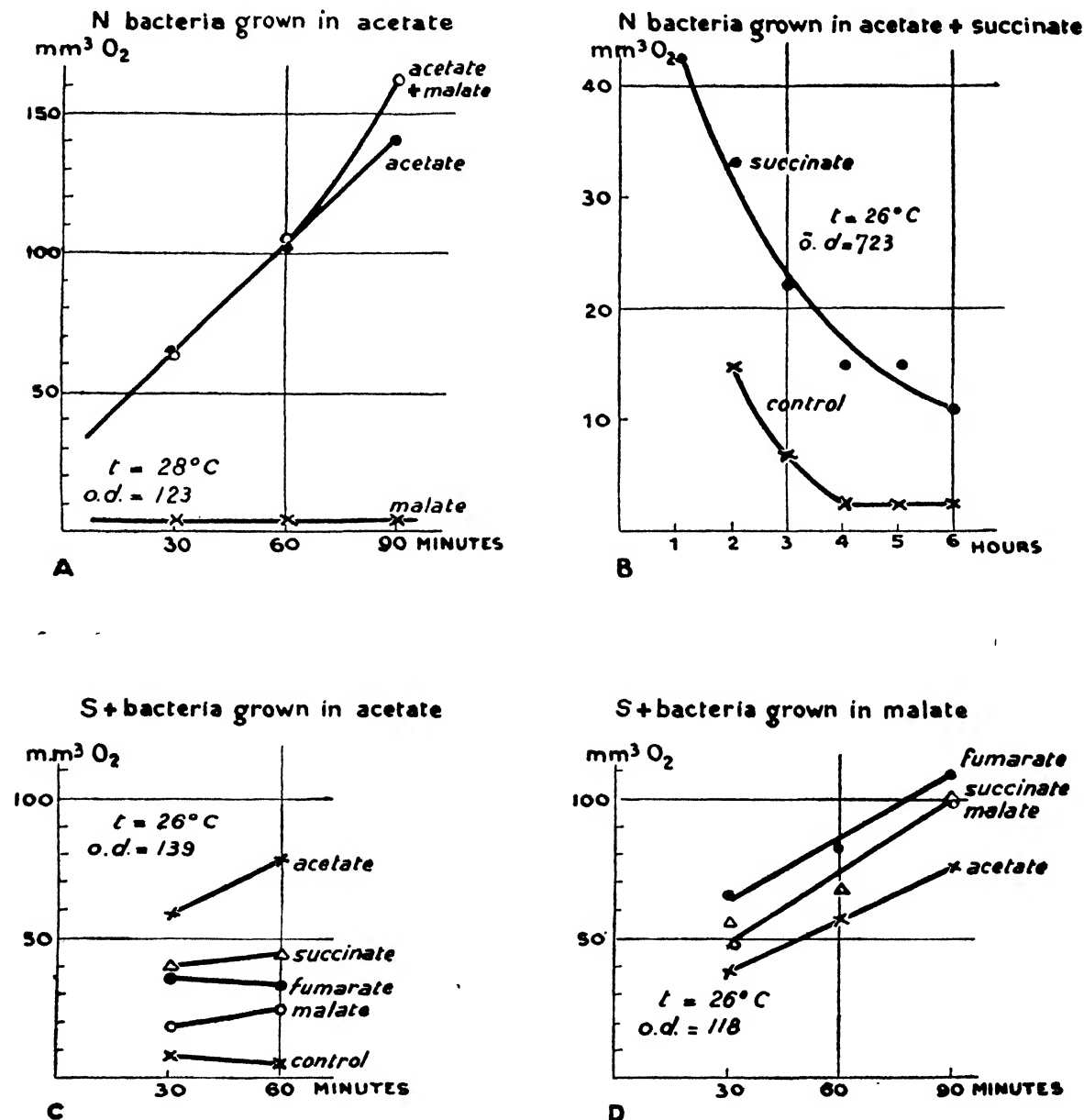


FIG. 1. Respiration of Moraxella. Oxygen consumption of 2 cm.³ of bacterial suspension measured in Warburg's manometer. o.d. = optical density as measured with Meunier's electrophotometer through 10 mm. of suspension. (100 units correspond to an absorption of .35 with λ = 0.55 microns.) A. Normal respiration and growth of $N$ bacteria. Absence of inhibitory action of malate. B. Respiration of $N$ bacteria (heavy suspension), showing small and decreasing oxidation of succinate. C and D. Oxygen consumption of $S^+$ bacteria. (A. Lwoff and A. Audureau, 26.)

however, seems to be ruled out by the following results: (1) In the presence of succinate, $S^+$ bacteria produce o.a.a. slowly and only after a negative period (Table 1). (2) The destruction of o.a.a. is almost entirely inhibited by succinate. (3) The production of o.a.a. is the same in the presence of malate or malate + succinate.

As the destruction of o.a.a. is inhibited by suc-

cinate, this means that malate itself inhibits the destruction of o.a.a. The same is true for fumarate. It appears that succinate does not inhibit the reactions, fumarate → malate, malate → oxaloacetate, which depend on different enzymes, but that the destruction of o.a.a. is inhibited by the three other $C_4$ diacids.

This seems to indicate that the four acids may
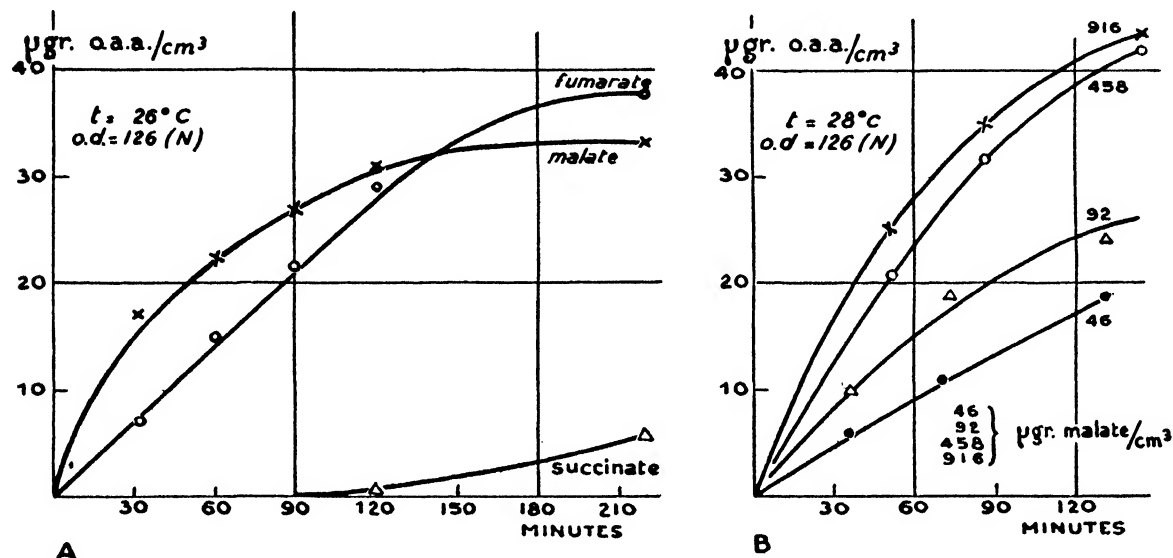


FIG. 2. Production of oxaloacetic acid (o.a.a.) by $N$ bacteria. o.d. = optical density (see Fig. 1). A. Unshaken culture. B. Shake culture. Varying concentrations of malic acid in mg./cm.$^3$ It is seen that $N$ bacteria oxidize $C_4$ dicarboxylic acids, but that the speed of o.a.a. production decreases rapidly. (A. Lwoff and A. Audureau, 26.)
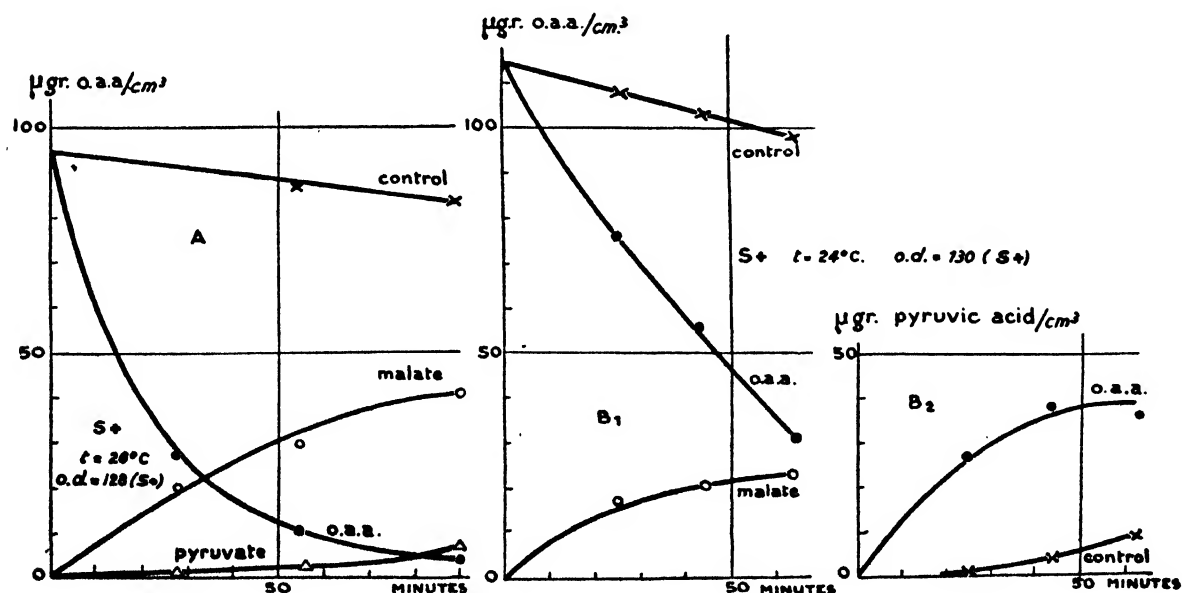


FIG. 3. Destruction of oxaloacetic acid (o.a.a.) by $S^+$ Moraxella. o.d. = optical density (see Fig. 1). Control = control without bacteria, or with boiled bacteria. A. Destruction of o.a.a. Production of o.a.a. from malate and from pyruvate. $B_1$. Destruction of o.a.a. Production of o.a.a. from malate. $B_2$. Production of pyruvic acid at the expense of o.a.a. (A. Lwoff and A. Audureau, 26.)

compete either for a single enzyme or for a common enzyme-precursor (see page 149).

*Destruction of o.a.a.; production of pyruvic acid.* O.a.a. was added to $N$ or $S^+$ bacteria and the remaining o.a.a. estimated by Straub's method (44) at regular time intervals. With $N$ bacteria, a small quantity of o.a.a. is consumed during the first hour. However, o.a.a. destruction soon stops. With $S^+$ bacteria, on the contrary, o.a.a. is consumed at a much higher rate, which shows no tendency to decrease except when the concentration of o.a.a. becomes very small (approximately 30 $\mu$ gr. o.a.a./cm.$^3$). (Fig. 4)

TABLE 1. PRODUCTION OF o.a.a. AT THE EXPENSE OF DIACIDS, WITH $S^+$ BACTERIA, AND INHIBITION OF o.a.a. DESTRUCTION BY SUCCINATE, MALATE, AND FUMARATE

|  | $\mu$gr. o.a.a./cm.$^3$ | $\mu$gr. o.a.a. destroyed |
|---|---|---|
| (A) o.a.a. control (1) | 182 |  |
| o.a.a. | 65 | 117 |
| o.a.a.+oxalate | 55 | 127 |
| o.a.a+citrate | 79 | 103 |
| o.a.a.+succinate | 178 | 4 |
| (B) o.a.a. control (1) | 113 | — |
| o.a.a. | 53 | 60 |
| o.a.a.+succinate | 109 | 4 |
| succinate | *0* | — |
| (C) succinate | *3.5* |  |
| fumarate | *22* |  |
| fumarate+succinate | *28* |  |
| malate | *34* |  |
| malate+succinate | *29.5* |  |
| o.a.a. control (1) | 91.5 | — |
| o.a.a. | 71.5 | 20 |
| o.a.a.+succinate | 94 | 0 |

(A) Estimation after 80 min. at 22°.
(B and C) Estimation after 70 min. at 25°.
(1) Control without bacteria. Concentration of o.a.a. at the end of experiments is given in the first column. Concentration of other diacids is 1 per 1000 (initial).
Numbers in italics: o.a.a. produced at the expense of diacids.

Thus, $N$ bacteria are able to destroy o.a.a., but the activity decreases rapidly as if the responsible enzyme were inhibited.

This inhibition might be attributed to the metabolic product of o.a.a. decarboxylation. It was proved, however, that pyruvic acid has no inhibitory effect on o.a.a. destruction. On the other hand, it was not possible to detect the production of inhibitory substances. $N$ bacteria, or the medium in which they have lived in the presence of o.a.a., do not inhibit the destruction of o.a.a. by $S^+$ bacteria. $N$ bacteria attack o.a.a. identically, whether suspended in a fresh solution of o.a.a. or in a solution where $N$ bacteria have ceased to destroy o.a.a. On the other hand, when centrifuged and suspended in a new solution, "poisoned" $N$ bacteria behave as "new" bacteria; that is, they seem to have been "detoxicated." But it is impossible to tell whether an inhibitory substance has been washed out or whether the enzyme has been allowed to "recover." It is difficult to draw any conclusion from these negative experiments. It will be seen further that some mutations are actually connected with the production of inhibitory substances.

According to classical conceptions, it seemed likely that o.a.a. was decarboxylated by $S^+$ bacteria. Actually, there is a production of pyruvic acid parallel to o.a.a. consumption. This was demonstrated through estimations by Straub's method (44) in acid medium. The experiments were performed in acid medium (pH 3.8) in the absence of nitrogen so as to prevent adaptation. The graphs in Fig. 3 ($C_1$ and $C_2$) show that during the first 30 minutes two-thirds of the o.a.a. decarboxylated by $S^+$ bacteria appears as pyruvic acid. That is to say, one molecule of pyruvic acid is produced for each molecule of o.a.a. destroyed.

The slow decarboxylation of o.a.a. by $N$ bacteria also yields pyruvic acid. It is thus possible to conclude that $N$ and $S^+$ bacteria are both able to decarboxylate o.a.a. and to yield pyruvic acid. But, whereas with $N$ bacteria the reaction is only started and stops very rapidly, with $S^+$ bacteria, on the contrary, it proceeds normally.

It seemed that the only way by which o.a.a. could yield pyruvate should be decarboxylation according to the equation:

$$COOH \cdot CH_2 \cdot CO \cdot COOH \rightarrow CO_2 + CH_3 \cdot CO \cdot COOH$$

oxaloacetic acid                   pyruvic acid

It was found, however, that *in anaerobiosis o.a.a. is not destroyed by $S^+$ bacteria.* Neither is o.a.a. destroyed in the presence of nitrate: the destruction of oxaloacetic acid appears to be dependent, directly or indirectly, on the presence of oxygen. Various hypotheses may be put forward to explain this:

(1) A high oxydo-reduction potential may be necessary for the reoxidation of a coenzyme-like substance taking part in the reaction (Moraxella is a strict aerobe).

(2) The decarboxylation may be preceded by an activation of the oxygens of the carboxylic groups. Szent-Gyorgyi (45) has already admitted a reaction of this kind for the oxidation of succinic acid.

(3) The first step in the attack of oxaloacetic acid could be a phosphorylation.

Reactions considered under (2) and (3) would be performed only in the presence of oxygen. The question remains entirely open.

*Conclusions.* The main points may be summarized as follows: the $N \rightarrow S$ mutation brings about a considerable increase in the activity of an enzyme (or enzymes) involved in the metabolism of the four $C_4$ dicarboxylic acids (succinic, malic, fumaric, oxaloacetic).

Since the activities of succinodehydrogenase, fumarase, and malicodehydrogenase appear to be about the same with $N$ and $S^+$ cells, it follows that

these enzymes are *not* involved in the $N \to S^+$ mutation.

Since, on the other hand, the destruction of oxaloacetic acid by $S^+$ bacteria is inhibited by the three other acids, it is clear that only a very small fraction, if any, of these can be metabolized according to the classical scheme: succinic $\to$ fumaric $\to$ malic $\to$ oxaloacetic $\to$ pyruvic.

being. It seems a good guess, however, that the postulated "first-step reaction" might consist of a phosphorylation. The hypothesis of phosphorylated intermediates of $C_4$ dicarboxylic acids has already been discussed by Stotz (*43*).

As $N$ bacteria are able to bring about a definite though slight destruction of oxaloacetic acid, it is at any rate highly probable that the $N \to S^+$ change
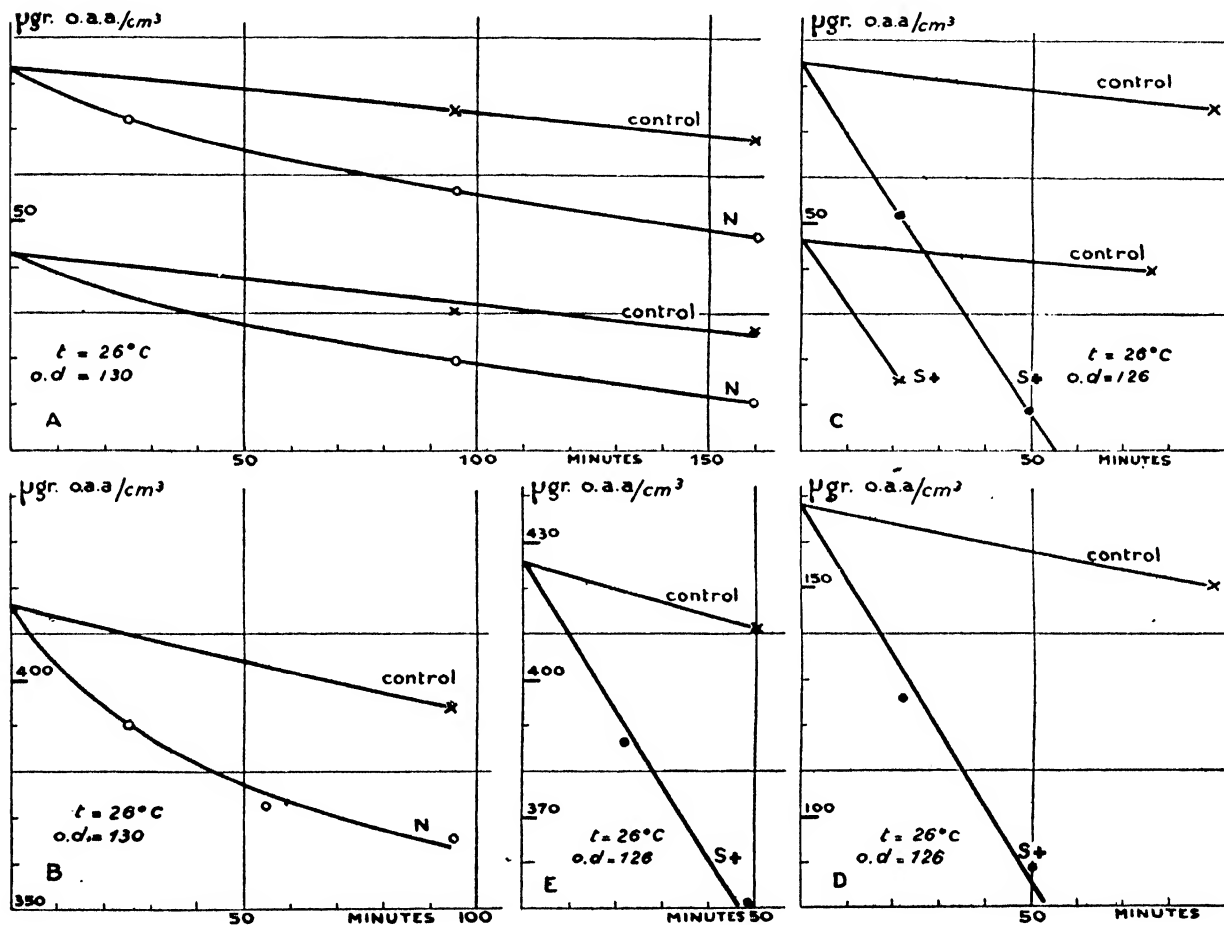


FIG. 4. Comparative destruction of oxaloacetic acid (o.a.a.) by normal ($N$) and mutated ($S^+$) Moraxella. o.d. = optical density of the bacterial suspension (see Fig. 1). (A. Lwoff and A. Audureau, *26*.)

This is further supported by the fact that oxaloacetic acid is not attacked in anaerobiosis, which shows that its decarboxylation must be preceded by another reaction, apparently dependent on oxygen. It seems reasonable to assume that the attack of the three other dicarboxylic acids occurs through the same "first-step reaction." Under this assumption, the $N \to S^+$ mutation would involve a change either in a single enzyme or in a common precursor to four specific enzymes catalyzing this reaction with the four acids.

The experimental evidence does not seem to warrant any more precise conclusions for the time

does not consist of the "*de novo*" synthesis of an enzyme. The preexisting "normal" enzymes as well as the "mutated" enzymes seem to be implicated in the *same chemical reaction*.

The $N \to S^+$ transformation apparently involves the "suppression of an inhibition." As other examples of such suppressions are known, it was considered convenient to coin the term "anaphragmic mutations" to designate them (from $\alpha\nu\alpha$ = contrary, $\varphi\rho\alpha\gamma\mu\sigma\varsigma$ = obstacle).

*Galactose mutation of Escherichia coli strain M.L.* The galactose mutation of *E. coli mutabile*, strain M.L., recently studied by Monod (unpub-

lished data) appears to belong to this type.

A study of the growth curves obtained with this strain in a synthetic medium, with various carbohydrates as sole carbon source, revealed that, whereas growth was perfectly normal with all other sugars, it was quite abnormal with galactose. After having started at a fairly normal rate (0.8 divisions per hour), growth suddenly slowed down, the rate dropping to about 0.35 divisions per hour (Fig. 5). $O_2$ consumption measurements in a Warburg apparatus gave similar results. It was thought that normal-growing bacteria might be selected by serial

seems to be known. No direct proof of this could be given, however.

Growth of $G^+$ and $G^-$ bacteria was compared in: (a) fresh galactose medium, (b) $G^+$ culture filtrate, (c) $G^-$ culture filtrate. It appeared that:

$G^-$ bacteria are inhibited by $G^-$ culture filtrates (initial growth rate 0.4 divisions per hour instead of 0.8 in fresh medium). (Fig. 6)

$G^-$ bacteria are only slightly inhibited in $G^+$ culture filtrates (growth rate 0.7 instead of 0.8 in fresh medium).

$G^+$ bacteria are not inhibited at all in $G^+$ culture
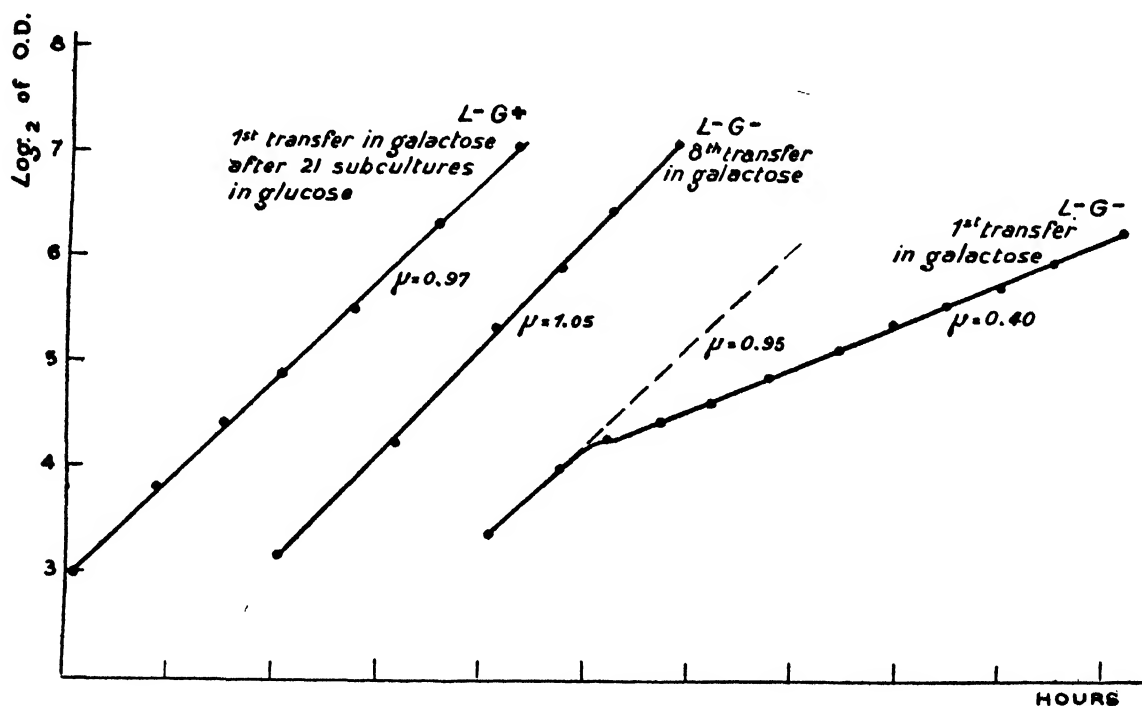


FIG. 5. Growth of *Escherichia coli* M.L. in a synthetic-galactose medium. (Semi-logarithmic coordinates.) Temperature 37° C. $\mu$ = growth rate. The curves show the abnormal growth of the original ($G^-$) strain and the normal growth of the selected ($G^+$) bacteria. (J. Monod, unpublished.)

transfers in galactose-synthetic medium. Actually, after 4 transfers (each involving 24-hour shake culturing at 37°), the two individual M.L. strains studied grew normally on galactose; 12 such transfers were performed, after which the two strains were plated out, and four new strains were started from isolated colonies. These four strains were subcultured through 25 transfers in glucose-synthetic medium. Measurements of growth on galactose after the 25th transfer showed that all four had retained their "normal" growth. We shall refer to these bacteria as $G^+$ and to the original strains as $G^-$. The stability of the $G^+$ strains makes it highly probable that the $G^- \rightarrow G^+$ transformation consists of the selection of a spontaneous mutant, rather than of "nonreversible adaptation," no example of which

filtrates (growth rate 1.05, as in fresh medium).

$G^+$ bacteria are only very slightly inhibited in $G^-$ culture filtrates (growth rate 0.95 instead of 1.05).

It was also shown that no such inhibitions ocurred, provided the medium contained *another carbohydrate* besides galactose. It thus appears that $G^-$ bacteria actually produce from galactose an inhibiting substance, which is secreted into the medium, and are highly sensitive to it. $G^+$ bacteria produce less of this substance, and are almost insensitive to the $G^-$ substance.

In this case, on account of the presence of the inhibiting substance in the medium, the "anaphragmic" nature of the $G^- \rightarrow G^+$ transformation is quite clear, and it seems probable that it belongs

to the same type as the Moraxella $N \to S^+$ mutation.

Other types of anaphragmic mutations seem to exist, examples of which may be found in the literature.

*Rhamnose mutation of Eberthella typhi.* The following observation was reported 35 years ago in the apparently forgotten work of Reiner Müller (*38*). *Eberthella typhi* plated on peptone agar + rhamnose gives very small colonies, as contrasted with the much larger size of colonies appearing on peptone agar *not* complemented with rhamnose. But numerous typical outgrowths appear, which, when re-



Fig. 6. Growth of $G^-$ *Escherichia coli* M.L. in fresh synthetic-galactose medium, and in $G^-$ culture filtrate. One o.d. unit corresponds to about $10^9$ cells per cm.$^3$ $\mu$ = growth rate. (J. Monod, unpublished.)

plated, give large normal colonies. Growth of the mutated strain is no longer inhibited by rhamnose.

The original strain is able to produce acid and gas from rhamnose, whereas the mutated strain does not show any such activity. Müller did not state whether either strain could use rhamnose as carbon and energy source. The original strain (which may be called rhamnose +) appears to be inhibited by rhamnose or by some metabolic product of rhamnose. The mutated strain has probably lost the power to attack rhamnose and to produce the inhibitory substance or substances.

The same rhamnose mutation was observed by Müller (*38*) with some Shigella (especially of the Flexner type)—not, however, with paratyphic or Gärtner bacilli. A short note indicates that an *arabinose mutation* of the same kind exists in some paratyphic bacilli.

*Xylose mutation of Salmonella "dublin."* A quite comparable case has recently been studied by Kris-

tensen (*17*), to whom we owe extensive work on mutations of fermentative abilities in the Salmonella. The growth of a normal *"Salmonella dublin"* strain is inhibited on xylose-peptone agar. But outgrowths from the small colonies may give large colonies which are no longer inhibited by xylose. The original strain produces acid from xylose, the mutated strain produces no acid and exhibits no xylose consumption. The xylose + as well as the xylose − strains are unable to utilize xylose as a carbon source in synthetic media. It does not appear from Kristensen's data whether growth is actually stopped or only slowed down in the presence of xylose. This remark holds for the rhamnose and arabinose mutations studied by Müller.

Interesting results might be expected from closer studies of this type of mutation. In these cases (as opposed to the Moraxella $N \to S^+$ and *E. coli mutabile* $G^- \to G^+$ mutations) it seems that some product of the metabolism of the active pentoses interferes with an essential reaction controlling growth, since the effect is apparent even in a complex medium. One is tempted to imagine that this may have something to do with the synthesis of an essential pentose such as ribose.

The interactions of isoleucineless and valineless mutants recently studied by Bonner may be considered as a model of suppressive action, analogous to the mechanism probably involved in some anaphragmic mutations. In *E. coli* (+), exogenous keto-valine (or valine) inhibits the amination of keto-isoleucine. In Neurospora, exogenous keto-isoleucine inhibits the amination of keto-valine. In the isoleucine −, valine + mutant of Neurospora (No. 16,117), the amination of keto-valine is possible, but not that of keto-isoleucine. As a consequence of the accumulation of keto-leucine, the amination of keto-valine may be prevented, leading to a secondary requirement for exogenous valine (D. Bonner, this Symposium).

*Conclusions.* The grouping of these different mutations under the designation "anaphragmic mutations" must of course be considered as provisional, and may well turn out to be artificial. From the little we know, however, "anaphragmic mutations" seem to be of general occurrence among various bacteria, and it is already possible to distinguish two types:

(1) In some cases the specific metabolic activity of the original bacteria brings about the inhibition of the enzyme concerned with the oxidation of the specific substrate itself, which is the carbon and energy source (*e.g.,* $C_4$ dicarboxylic acids, galactose). The mutation allows the specific substrate to be normally attacked.

(2) In other cases, the mutation appears to suppress the attack of the specific substrate and thus the production of substances inhibiting some important growth reaction not necessarily related to the specific substrate.

*Interrelations between Mutation and Adaptation*

*Lactose mutation of Escherichia coli mutabile.*
Enzymatic adaptation involving melibiozymase and galactozymase has been proved, in yeast, to be gene-controlled (*19*). No such demonstration could of course be given with bacteria. Changes in enzymatic properties of a bacterial strain have been described either as mutations or as adaptations, since definite proofs of the occurrence of each of these phenomena seem always to have come from different, distinct cases. There are good reasons to believe, however, that many such changes of enzy-

piration of both is equal to that of controls without substrate. On the other hand, $L^+$ bacteria grown in *lactose* have a normal respiration on lactose (Fig. 7). Their $O_2$ consumption is of the same order of magnitude as in glucose and remains quite constant through the experiments.

These results show that the lactose enzyme of $L^+$ bacteria is a *strictly adaptive enzyme* in Karstrom's (*15*) sense.

This was confirmed by growth measurements. It must be recalled here that analysis of the phenomenon of "diauxis" (*29-31*) has shown that
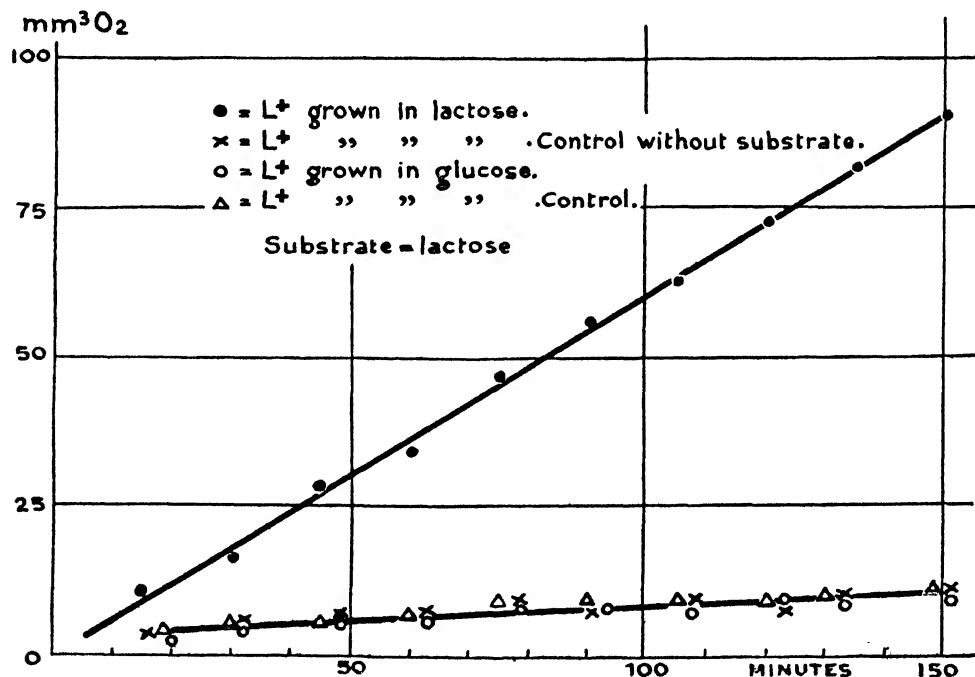


FIG. 7. $O_2$ consumption of adapted and nonadapted $L^+$ *E. coli* M.L. with M/25 lactose. Optical density (see Fig. 6) of suspensions = 480. The rate of $O_2$ uptake with $L^+$ bacteria grown in *glucose* is not greater than that of the control. $O_2$ consumption by $L^+$ bacteria grown in *lactose* is seen to be much greater. (J. Monod and A. Audureau, *37*.)

matic properties may involve the functioning of *both* mechanisms. A clear example of this is the lactose mutation of an *E. coli mutabile* strain recently studied by Monod and Audureau (*37*). This strain (M.L.) is a typical mutabile as described by Massini (*28*), Marchal (*27*), and Lewis (*18*).

Over ten $L^+$ clones were isolated from the original $L^-$ strain, all of which proved to be quite stable. Even after 25 transfers in *glucose* synthetic medium, they gave rise exclusively to $L^+$ colonies, when plated on synthetic lactose agar.

Measurements of $O_2$ consumption by $L^+$ and $L^-$ bacteria in lactose were performed in presence of 2-4 dinitrophenol, which is known to inhibit synthesis (*10*) and enzymatic adaptation (*34*). It was found that $L^+$ bacteria grown in *glucose* do not attack lactose any more than $L^-$ bacteria. The res-

adaptation of all specific *adaptive* enzymes attacking carbohydrates in bacteria is inhibited by other carbohydrates, which are attacked by *constitutive* enzymes. When bacteria are grown in a synthetic medium with a mixture of two carbohydrates, one of which is attacked by an adaptive, the other by a constitutive enzyme, growth is characterized by two complete growth cycles, separated by a lag period. Regardless of which substrate the bacteria may have been adapted to previously, the first growth cycle always corresponds to assimilation of the "constitutive substrate," the second to the "adaptive substrate."

When $L^+$ bacteria, previously grown on lactose, are inoculated into a lactose-glucose medium, the typical "diauxic" growth curve is obtained, whereas growth of $L^-$ bacteria is of course limited to the

first cycle, corresponding to the assimilation of glucose (Fig. 8). It is seen from the growth curve that the suppression of lactose adaptation is quite complete. A definite lysis, characteristic of the absence of an assimilated carbon source (30-31) occurs during the lag period separating the two growth cycles.

These results show that gain by $L^-$ bacteria of the ability to attack lactose involves the functioning of two mechanisms, one of which is spontaneous and irreversible, the other substrate-induced and entirely reversible. $L^-$ bacteria do not seem to differ from $L^+$ bacteria by the possession of a "new" en-

ready mentioned above. Such interactions become apparent when the $G^- \rightarrow G^+$ transformation is followed comparatively with $L^+$ and $L^-$ clones.

For instance, in one experiment four clones, two $L^-G^-$ and two $L^+G^-$, all of which had previously been grown in glucose, were transferred to galactose-synthetic medium. Growth measurements were performed at the 1st, 4th, and 8th transfers. The following table gives the values of the two successive growth rates for each clone at these successive stages. The letter "N" means that growth is normal (one growth rate instead of two).
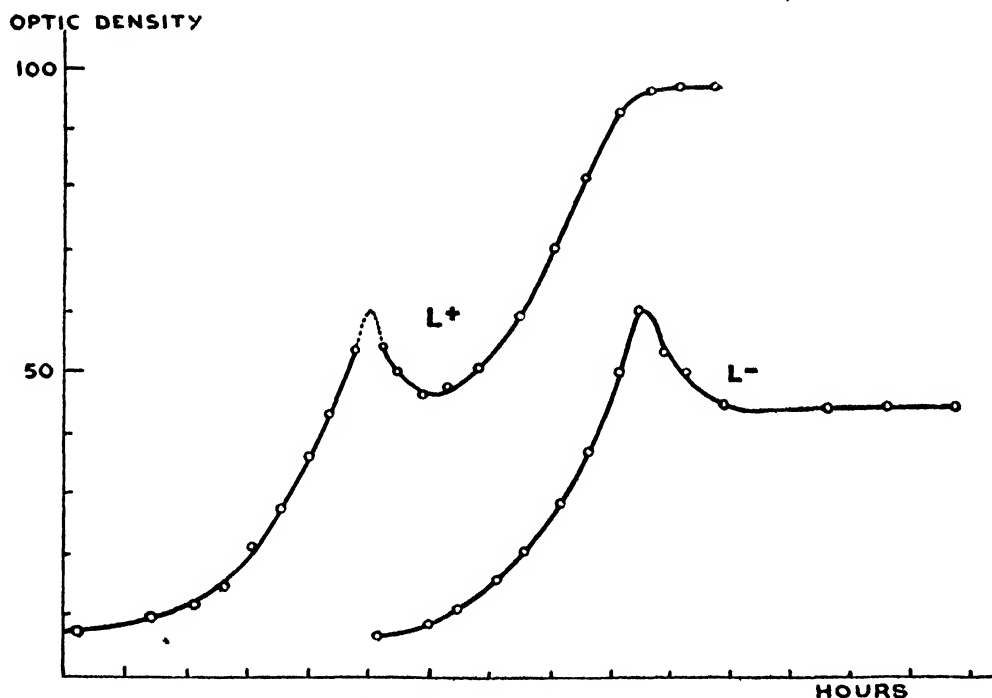


FIG. 8. *Growth of $L^-$ and $L^+$ Escherichia coli mutabile* on a mixture of glucose and lactose (1 per 10,000 each.) o.d. = optical density (see Fig. 6). $L^-$ bacteria utilize only glucose. $L^+$ bacteria show the typical "diauxic" curve: (1) utilization of glucose, (2) lysis due to inanition, (3) adaptation and utilization of lactose. (J. Monod and A. Audureau, 37.)

zyme, but rather by the faculty of adaptation of a precursor. The results further show that adaptation of this precursor may be inhibited or suppressed by other carbohydrates, such as glucose, the attack of which however depends on *another specific enzyme*. This appears in particular from the fact that both $L^-$ and $L^+$ bacteria attack glucose with practically equal intensity.

The question of the mechanism of these adaptations and inhibitions is of first importance to our understanding of the nature of mutations of the $L^- \rightarrow L^+$ type.

*Interactions between the lactose and galactose mutations of E. coli mutabile strain M.L.* Before discussing this, it may be useful to state a few facts concerning interactions between the $L^- \rightarrow L^+$ mutation and the $G^- \rightarrow G^+$ (galactose) mutation, al-

It is seen that:

(a) On the initial transfer the second growth rate is higher with $L^+G^-$ bacteria than with $L^-G^-$ bacteria.

(b) The $G^- \rightarrow G^+$ transformation (selection of $G^+$ bacteria) takes place much more rapidly with $L^-$ clones than with $L^+$ clones. This difference is very striking at the 4th transfer (Fig. 5).

In another experiment, one $L^+G^-$ and one $L^-G^-$ clone were subcultured through 12 transfers in galactose, and plated out on agar. Three colonies of each were picked, and transferred to glucose-synthetic medium. Growth measurements on galactose were made after the 1st and 25th transfers in glucose. It appeared that the 3 $L^-G^+$ clones derived from the original $L^-G^-$ clone gave perfectly normal growth curves, whereas the $L^+G^+$ clones derived

from the $L^+G^-$ gave growth curves of a distinctly different type, with two successive periods of equal growth rate, separated by a short period during which growth was slightly slower (Fig. 9). The same results were obtained when this experiment was repeated with two other $L^+$ and $L^-$ clones.

These observations seem to show that the $L^+ \to L^-$ mutation brings about slight but definite changes of the properties of the galactose-attacking enzyme, although lactose and galactose are attacked by undoubtedly different, specific enzymes.

specific enzyme, or a precursor. None of these cases, however, seems to be adequately described as a loss or a *de novo* synthesis of a "new" enzyme structure. In the Moraxella $N \to S^+$, as well as in the $G^- \to G^+$ mutation of *E. coli*, it is highly probable that the change results in "normalizing" the functioning of an enzyme which is already *present* in the original $N$ and $G^-$ cells. Very few data are available for the time being about the specific metabo-
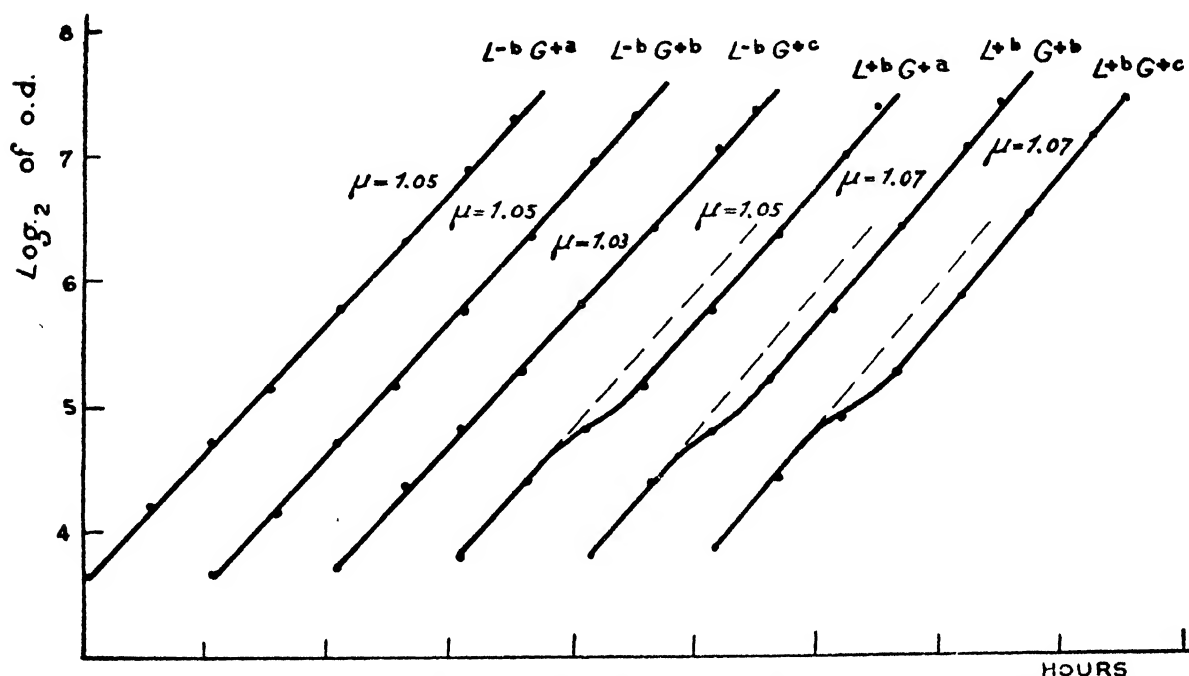


Fig. 9. Growth of 3 $L^-G^+$ and 3 $L^+G^+$ strains on galactose-synthetic medium. (Semi-logarithmic coordinates.) Note distortion of growth curve with $L^+G^+$ strains as compared to $L^-G^+$ strains. (J. Monod, unpublished.)

TABLE 2. GROWTH RATES (GIVEN AS DIVISIONS PER HOUR) OF $L^-G^-$ AND $L^+G^-$ CLONES ON SUCCESSIVE TRANSFERS IN GALACTOSE-SYNTHETIC MEDIUM

| Transfer No. | I Growth rates | | IV Growth rates | | VIII Growth rates | |
|---|---|---|---|---|---|---|
| | 1st | 2nd | 1st | 2nd | 1st | 2nd |
| $L^-G^-$ clones {A | 0.86 | 0.37 | 0.95 | N | 1.0 | N |
| {B | 0.80 | 0.42 | 0.95 | N | 1.0 | N |
| $L^+G^-$ clones {A | 0.80 | 0.48 | 0.77 | 0.56 | 1.0 | 0.95 |
| {B | 0.80 | 0.50 | 0.80 | 0.56 | 1.0 | 0.90 |

enzyme, although lactose and galactose are attacked by undoubtedly different, specific enzymes.

### Discussion

*Bacterial mutations as variations of enzyme structures.* It is quite clear that in all cases studied above, the mutational change appears to involve a

lism of "normal" and "deficient" strains. It seems likely that many more examples of "anaphragmic" mutations may be revealed by such studies, which are highly desirable.

In the case of the $L^- \to L^+$ mutation it is reasonable to assume that the change consists of a structural modification of a precursor, which makes it "adaptable" to lactose. This, however, brings up the problem of the mechanism of enzymatic adaptation, which is obviously of greatest importance to our understanding of enzymatic mutations.

Yudkin (47) put forward the hypothesis of a mass-action equilibrium between each enzyme and a precursor. However, it should be pointed out that no hypothesis can be considered satisfactory unless it accounts for the following properties of enzymes attacking carbohydrates in bacteria: (a) specificity, (b) *specific* character of enzymatic adaptation, (c) *nonspecific* suppressing effect expressed in the phenomenon of diauxis, (d) nonadditivity of activities.
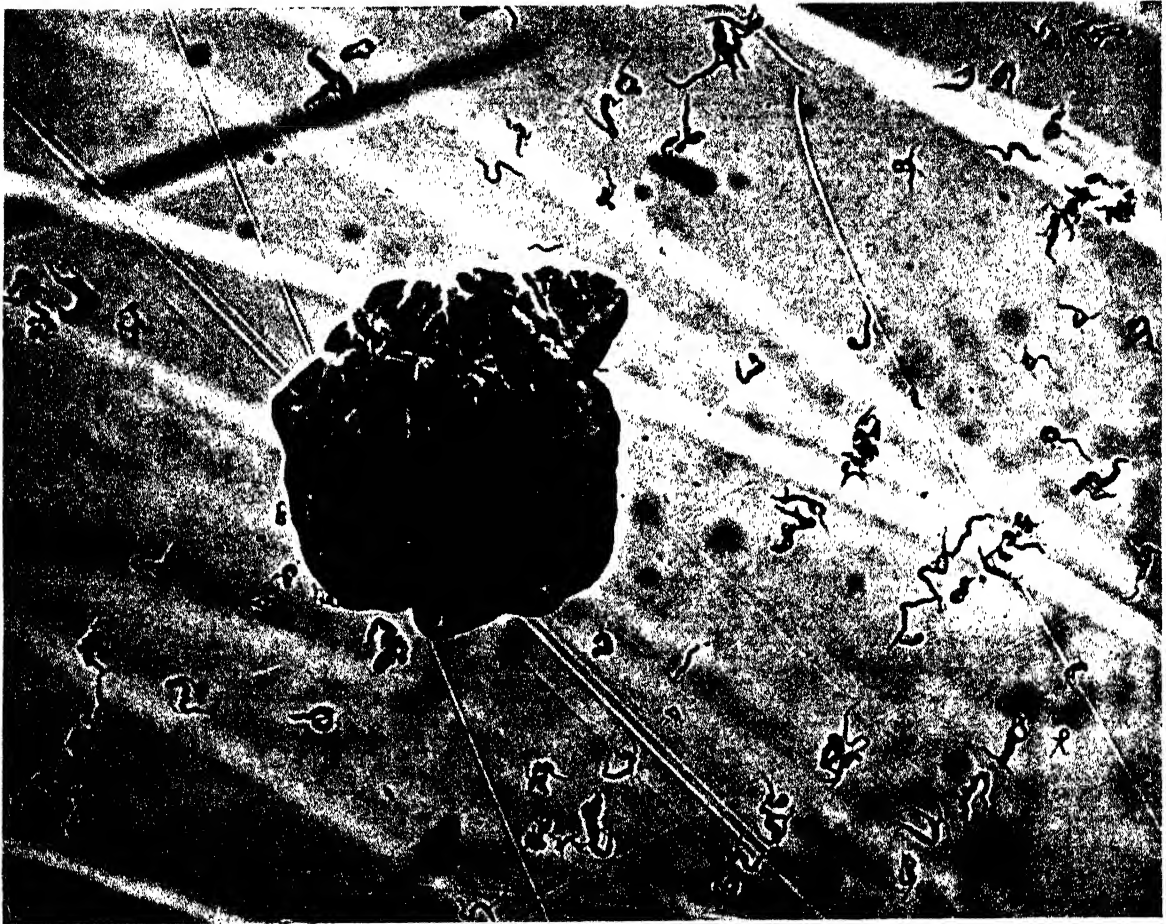
Fig. 10. Photograph of colonies appearing on synthetic agar medium plated with a "normal" broth culture of strain L.A. The photograph shows numerous, minute, vermicular ".N" colonies, and one large "M" colony. (J. Monod, 36.)

The following working hypothesis has been advanced by Monod (*32-35*).

Enzymes allowing attack of carbohydrates by bacteria are all derived from a *common precursor*. This precursor (preenzyme) has a slight general affinity for carbohydrates. Transformation of the precursor into an adapted, specific enzyme occurs as a result of the substrate-preenzyme combination (which would account for the specificity of enzymatic adaptation).

Certain substrates might have greater affinity for the preenzyme and thus be able to displace other substrates from it, which would explain the *nonspecificity* of the inhibiting effect of "constitutive" substrates.

Interactions such as have been disclosed between the galactose and lactose mutations seem to agree well with the view that enzymes attacking carbohydrates may stem from a common precursor. It is quite clear that any modifications occurring in the precursor molecule might be expected to interact with each other. The facts concerning the $N \rightarrow S$ mutation of Moraxella might also be explained on the basis of a structural change involving a common precursor to four specific enzymes.

It must be pointed out that basically similar hypotheses were suggested by Emerson (*13*) to explain enzymatic adaptation. However, Emerson's main object was to explain *gene-controlled* adaptation as disclosed by Spiegelman and Lindegren's (*19, 40-42*) highly interesting results on yeast. This leads us to the weak point in bacterial genetics. Spiegelman and Lindegren's researches have shown that while enzymatic adaptation of yeast melibiozymase is gene controlled, the enzyme (or its precursor) is not to be identified with the gene. Now, is there any way of testing this with bacteria? Is there any point, pragmatically, in raising the question?

The problem of bacterial genes was discussed as early as 1912 by Beijerinck (*6*), who admitted the existence of "progenes" or latent genes able to mutate into active genes. Beijerinck also put forward the hypothesis that enzymes could represent the genes themselves. After 35 years, however, this problem is still unsolved.

We hope that the following considerations will make one feel a little less pessimistic than is usual about this rather irritating question.

Let us consider the $G^- \rightarrow G^+$ and $L^- \rightarrow L^+$ mutations of *E. coli*, each concerning the activity (or adaptability) of two specific carbohydrate-attacking enzymes, $E_g$ and $E_l$. If we assume that each derives from a precursor ($P_g$, $P_l$) and that this precursor is *distinct* from the gene ($G_g$, $G_l$) controlling its structure, we may consider the following possible schemes of gene-controlled enzyme synthesis:

$$G_g \text{-----------} P_g \longrightarrow E_g \atop G_l \text{-----------} P_l \longrightarrow E_l \qquad (1)$$

$$G_g^- \text{-------} P_{gl} \longrightarrow E_g \atop G_l^- \text{-------} \qquad E_l \qquad (2)$$

$$G_{gl} \text{---------} P_{gl} \longrightarrow E_g \atop E_l \qquad (3)$$
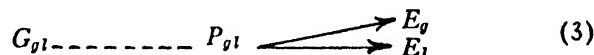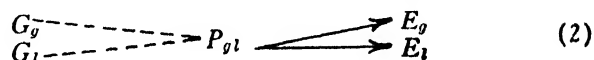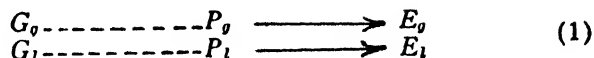
Scheme no. 1 may be dropped, since it does not account for any interactions between the two mutations, nor for the diauxic interrelations between carbohydrate-attacking enzymes.

This, on the contrary, is accounted for by schemes no. 2 and no. 3. Scheme no. 3 might be considered more likely than no. 2 on the "one gene-one enzyme" hypothesis strongly advocated by Beadle (*5*) and brilliantly supported by his own and his co-workers' results on Neurospora. (In our case "one gene-one enzyme" would be replaced by "one gene-one precursor.") Scheme no. 2 cannot be rejected *a priori*, however, especially since there may be a method of testing its value.

Admitting scheme no. 3 is equivalent to assuming that the various possible combinations of *g* and *l* characters each correspond to a different allelomorph of gene $G_{lg}$:

$$G_{l-g-} \qquad\qquad G_{l+g-}$$
$$G_{l-g+} \qquad\qquad G_{l+g+}$$

It should then be considered very unlikely that the mutations $G_{l-g-} \rightarrow G_{l+g-}$ and $G_{l-g+} \rightarrow G_{l+g+}$ should be characterized by identical mutation rates; and the same would of course be true of mutations $G_{l-g-} \rightarrow G_{l-g+}$ and $G_{l+g-} \rightarrow G_{l+g+}$, especially since *both allelomorphs* would be different in each case.

It is obvious, on the contrary, that the *same transformations* would be expected to occur with *identical* mutation rates if the common precursor were controlled by two different, independent genes ($G_g$ and $G_l$, scheme no. 2) even though there might be interactions in the *results* of these *independent* mutations.

Confirmation of scheme 2 would at the same time prove that the precursor is actually distinct from the genes. If scheme 3 were confirmed, the question would remain unsettled, though it would of course bring strong support to the one gene-one precursor (or enzyme) theory.

This test has not been applied to the $G$ and $L$ mutations, owing to technical difficulties in estimating the mutation rates. However, we thought it might be interesting to state this problem fully, since it is a good example of the difficulties that confront bacterial genetics and of possible ways of attacking them. Delbrück (*11*) has already emphasized that any knowledge we may acquire about bacterial "genes" must come from the study of *mutability* and mutation *rates*. Demerec and Fano (*12*) have given a good illustration of this with their study of phage-resistant mutants.

Further progress will undoubtedly be made by close, *quantitative* studies of mutant strains (involving identification and isolation of the enzymes concerned), *combined* with the development of methods, techniques, and material for the estimation of bacterial mutation rates.

## GROWTH-FACTOR MUTATIONS

Variations of growth requirements among bacteria have been known for a long time, in fact long before the concepts of growth factors and essential metabolites were clearly defined. However, very few cases of such variations have been studied in such a way as to allow a conclusion regarding their *adaptive* or *mutative* nature.

Two cases will be described here, both of which involve a change in an amino-acid requirement.

### Methionine Mutation of a Coliform Bacterium

The simplest case is that of a bacterium of the coli-aerogenes group (strain L.A.) recently studied by Monod (*36*). This strain develops readily when inoculated into liquid synthetic medium. When broth cultures are plated on synthetic-glucose-agar, however, an enormous majority of very minute (less than 0.1 mm.) "*N*" colonies develop. Among these are a few large "*M*" colonies (Fig. 10). Isolation of *N* and *M* colonies, with subsequent subcultures on peptone broth and replating on glucose-synthetic agar, showed that *N* colonies give rise to *N* strains, which always contain a small number of *M* bacteria. *M* colonies give pure *M* strains, which remain stable even after 25 subcultures on peptone broth. Inoculation of *N* strains in liquid synthetic medium promptly results in selection of *M* bacteria, giving only *M* colonies on synthetic agar.

It is obvious that the "normal" (*N*) form is unable to synthesize an essential metabolite, present in peptone, though it may give a spontaneous mutant (*M*) which is able to do so. This essential metabolite was identified as methionine. *N* strains plated on synthetic-agar medium complemented with methionine, yield only large *M*-type colonies. None of the 17 other amino acids that were tested exerts any activity, whereas methionine is quite active at dilutions up to M/400,000. *dl*-methionine exerts no visible effect on the size or aspect of *M* colonies.

*N* strains may be subcultured on synthetic medium complemented with methionine, through apparently any number of transfers, without *M* bacteria being selected. This appears to furnish good proof that the *N* → *M* mutation is linked only with methionine synthesis, not with any other essential metabolite. The proportion of *M* bacteria in glucose-methionine-synthetic medium is about the same as in broth cultures; that is, about 1 *M* to 2500 *N*.

Growth measurements were performed with *N* and *M* clones, in glucose-methionine-synthetic medium at 37°. Growth rates during the exponential

phase correspond to 1.45 divisions per hour for the *N* clone, 1.05 for the *M* clone. From these data, a tentative calculation indicates the probability of the *N* → *M* mutation to be about 0.0001 per bacterium per division cycle.

These observations represent a clear-cut case of a change of synthesizing abilities occurring through spontaneous mutation. The fact that the acquisition of the power to synthesize methionine should be associated with a 36% decrease in growth rate seems noteworthy, and will be referred to later in the discussion.

### Proline and Phage-Resistance Mutations of *Escherichia coli*

The next case to be studied here appears much more complex, since it involves both a change of synthesizing ability and a change of sensitivity to a phage. The work of Burnet (*8, 9*) has shown that among intestinal bacilli resistance to a phage, or to certain types of phage, may frequently be associated with certain antigenic structures. From a keen analysis of these relations, Burnet concluded that phage-resistant variants were spontaneous mutants. This has now been proved beyond doubt by Luria and Delbrück's (*20*) statistical analysis.

According to Anderson (*1*), these mutations may be associated quite frequently with a loss of synthesizing abilities. However, in none of the cases studied by Anderson could the essential metabolites involved be identified.

This problem has recently been reinvestigated by Elie Wollman (*46*). An *E. coli* strain (C.B.) was used, which grew readily on synthetic media not complemented with growth factors. Twelve different phage-resistant mutant strains were isolated through selection with various phages. Five of these strains were no longer able to grow readily on synthetic media. Two of the five deficient mutants (*M₁* and *M₂*) were studied. Seventeen amino acids and six vitamins were tested for growth-promoting activity with $M_1$ and $M_2$. In both cases, the only active substance proved to be *l*-proline. None of the other substances exerted any activity. Strains $M_1$ and $M_2$ grew readily on glucose-*l*-proline-synthetic medium. It was found that *l*-proline was still active at a concentration of $M/10^8$.

It soon appeared, however, that strains $M_1$ and $M_2$ could also give positive cultures in synthetic media *not* supplemented with proline, provided the tubes were kept long enough (from 4 to 8 days) in the incubator. Further experiments showed this to be due to selection of a spontaneous mutant. The mutants could be demonstrated in cultures plated out on synthetic-agar medium.

Isolation of these proline-positive mutants as pure stable strains proved to be exceedingly difficult. Two methods were used: (1) selection by serial transfers in liquid synthetic media *without proline*, and (2) serial isolation and replatings of

proline-positive colonies appearing on synthetic agar. Even after 20 transfers on synthetic medium, followed by plating out on synthetic agar, strains $M_1$ and $M_2$ still yielded a high proportion (about 50%) of proline-negative colonies. Isolation of "proline-positive" colonies with immediate replating also gave a mixture of both types, often with a majority of negatives. Eventually, by combining both methods, stable proline-positive clones were isolated. These retained their "pure proline-positive" character after 20 transfers in broth or glucose-proline-synthetic medium. These strains ($P_1$ and $P_2$) were compared with $M_1$ and $M_2$ and with the original (C.B.) strain, as to cultural characteristics and sensitivity to a limited range of phages.

The characters of each form with regard to proline-synthesis and sensitivity to 4 phages are summarized in Table 3.

TABLE 3. SYNTHESIS OF PROLINE AND RESISTANCE TO PHAGES IN ESCHERICHIA COLI STRAIN C.B. AND MUTANTS DERIVED FROM IT

| Strains | Sensitivity to phages | | | | Proline synthesis |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| CB | + | + | + | + | + |
| $M_1$ | 0 | 0 | + | 0 | 0 |
| $P_1$ | 0 | 0 | + | 0 | + |
| $M_2$ | 0 | 0 | + | + | 0 |
| $P_2$ | 0 | 0 | + | + | + |

(After Elie Wollman, 46.)

It is seen that: (a) $M_1$ and $M_2$ differ from each other in phage sensitivity. (b) $P_1$ and $P_2$ appear to have *retained* the phage-resistance characters of $M_1$ and $M_2$ respectively.

The most likely interpretation seems to be that:

(1) $M_1$ and $M_2$ are derived from C.B. through independent spontaneous mutations, both of which involve at the same time a loss of sensitivity to several phages, and a loss of the power to synthesize $l$-proline.

(2) $M_1$ and $M_2$ are both able to mutate to a proline-synthesizing form. This is *not a true reversion*, however, since both $P_1$ and $P_2$ differ from C.B. as regards sensitivity to several phages.

(3) $P_1$ and $P_2$ are highly unstable and revert to $M_1$ and $M_2$ with a high rate.

(4) Stable forms of $P_1$ and $P_2$ may appear, but it is not clear whether they are derived from $P_1$ and $P_2$ or directly from $M_1$ and $M_2$.

## Discussion

*Phage-resistant mutants and growth-factor deficiencies.* Out of 800 clones isolated from X-ray-treated *E. coli* cultures by Gray and Tatum (14), only two were deficient mutants. With *untreated* *E. coli* cultures, the proportion of deficient clones found by Roepke, Libby, and Small (39) was only 6 out of 1994.

These data, when compared to Anderson's (1) (27 deficient strains out of 58 phage-resistant mutant strains) and Wollman's (5 out of 12), seem to rule out completely the hypothesis that in these cases phage resistance and growth-factor deficiencies may represent chance summation of independent variations. No data are available allowing a precise statistical treatment of the problem; but it seems obvious that in most, if not all, of these cases the two characters are closely associated and probably represent single mutations. On the other hand, both Anderson's and Wollman's results indicate that, even among mutant clones derived from a single strain through the selective action of a single phage, some may be growth-factor deficient and others not. This means that there is no *direct* physiological link between the two characters (i.e., that one is not the cause of the other). Consequently, an explanation of the fact that the two types of change may be associated must be sought further back.

The following type of scheme, adopted by Anderson, could account for it.

$$A \xrightarrow{\ G_1\ } B \overset{\displaystyle G_2}{\underset{\displaystyle G_3}{\diagdown\!\!\diagup}} \begin{array}{l} S_\varphi \\[2ex] M \end{array}$$
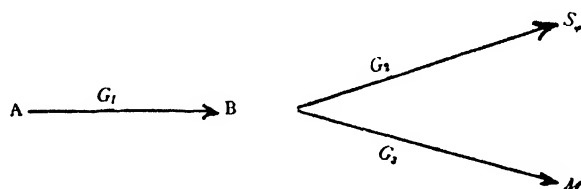
M is an essential metabolite, $S_\varphi$ a specific substance responsible for sensitivity to a given phage. The synthesis of both is assumed to depend on a common precursor B. Each metabolic step (*e.g.*, $A \to B$; $B \to S$; $B \to M$) might be interrupted as the result of mutations of "genes" $G_1$, $G_2$, $G_3$. Such a scheme may easily be adapted to account for results involving synthesis of several metabolites and phage-sensitizing substances.

Wollman's findings show, however, that some mutants may actually *"regain"* a synthesizing ability without regaining the phage sensitivity which had been *lost with it*, apparently as the result of a single mutation. This appears from the following scheme:

$$CB(P^+\ \varphi_1^+) \longrightarrow M(P^-\ \varphi_1^-) \longrightarrow P_1(P^+\ \varphi_1^-)$$
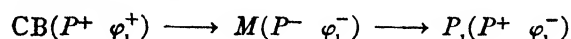
representing the sequence of changes involved in production of mutants $M_1$ and $P_1$ from the original strain ($P^+$ indicates ability to synthesize proline, $\varphi_1^+$ sensitivity to phage $\varphi_1$). Under the assumption made above, the $M_1 \to P_1$ transformation could only result from simultaneous mutations of two genes, which is most unlikely. It seems more reasonable to assume that in these cases an alternate

synthesis of proline, controlled by another gene, is possible. Obviously, other hypotheses are equally possible; for instance, that both syntheses may be controlled by a single gene, which could exist in various allelomorphic states. Here again, it appears most regrettable that bacteria should be devoid of sexual reproduction.

Even though no precise explanations can be brought forward for the time being, the correlations disclosed by Anderson's and Wollman's findings seem highly significant. Progress in that direction may be expected to bring important results. Another line of attack on this problem should also be tried: this would be to choose a number of "normal" and spontaneous biochemical mutant strains, and test them systematically against a large range of phages.

*Growth-factor mutations and evolution of bacteria.* It is now a firmly established fact that parasitic microorganisms are all characterized by much lower synthesizing abilities than free organisms. Neither is there any doubt that these "poor synthesizers" have derived from free "high synthesizers," through a succession of losses of functions, which have become more or less stabilized. (For a general survey of this problem see Lwoff, *22*.)

It seems reasonable to assume that this evolution resulted from selection of spontaneous mutants, though very few variations of synthesizing abilities have been actually *proved* to have occurred through spontaneous mutations. The only adequately described spontaneous growth-factor mutations appear to be Koser and Wright's (*16*) nicotinamide mutation of Shigella, Monod's (*36*) methionine mutation of Aerobacter (both of which are in fact *gains* of functions), and Roepke, Libby, and Small's (*39*) deficient strains of *E. coli*.

The mere fact that parasites have in almost all cases lost quite a number of functions proves that such "losses" may actually bring a selective advantage, provided the medium contains enough ready-made metabolites. The question is whether this advantage is a result of the loss of synthetic ability as such or of some associated change. Nevertheless, it is indeed quite conceivable that an organism may have an advantage in not performing certain syntheses. This might lead to an acceleration of some other essential growth reactions—that is, to an *increase in growth rate*. The fact that acquirement of the power to synthesize methionine by Aerobacter is associated with a 36% *decrease* in growth rate is, of course, no proof of this. But if constant correlations of this kind should be detected with other mutant strains, the hypothesis would become likely.

Analysis of this phenomenon might throw some light on the mechanism of evolution of microorganisms, and perhaps not of microorganisms alone.

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## DISCUSSION

Zamenhof: There are publications indicating that in a lactose-negative strain of *E. coli mutabile* there is actually a lot of lactase present, almost as much as in the lactose-positive strain, and that a lactose-negative strain cannot ferment lactose merely because lactose cannot penetrate into the cell. I am sure this complication has been excluded in Dr. Lwoff's and Dr. Monod's work, but in general one has to bear in mind the possibility of mutations in selective permeability of the cell membrane whenever one deals with intracellular enzymes.

Lwoff: Dr. Zamenhof has raised the question whether adaptive and mutative phenomena in bacteria can be explained by "permeability" effects, citing the results of Deer and co-workers on *Escherichia coli mutabile*. This question raises the problem of the nature of enzymes responsible for the primary attack of organic carbon sources. Numerous papers on bacterial mutations deal with changes in the power to attack sugars or other compounds or to utilize them as carbon or energy sources. In no case has the responsible enzyme been identified. It must be emphasized, however, that, whereas very little indeed is known about the aerobic attack of glucides by bacteria, many facts indicate that the attack of disaccharides does not involve a preliminary hydrolysis. These data have been recently reviewed by Leibowitz and Hestrin. Recent results of Doudoroff and of Leibowitz and Hestrin indicate that the first step in the attack of disaccharides may be phosphorylation. J. Monod and M. Morel have shown that the entire molecule of saccharose is utilized by *Proteus vulgaris*, but that "free" fructose, either alone or in a mixture of glucose and fructose, is not utilized at all. This result may be understood if we admit that Proteus is (1) unable to phosphorylate fructose, (2) able to phosphorylate saccharose and to split phosphorylated saccharose into glucose and phosphorylated fructose which may be utilized. The "common precursor" could be the common precursor of specific phosphorylases.

On the other hand, Dr. Spiegelman (J. Bact. 49: 108) has recently shown that yeast is able to oxidize sugars before it is adapted to ferment them. But the rate of oxidation is very slow and allows only a very slow and limited growth. Moraxella shows a quite similar picture. The normal and the mutant strains dehydrogenate $C_4$ dicarboxylic acids at the same speed, but the reaction stops, probably when the equilibrium is reached between the oxidized and reduced substance. This reaction is unable to support normal respiration and growth,

which are performed at the expense of dicarboxylic acids only by the mutated bacteria. $C_4$ dicarboxylic acids, as well as sugars, are apparently metabolized by two different pathways. One is a purely oxidative mechanism and is unable to support growth. The other probably involves a phosphorylation and is the only mechanism allowing normal metabolism and growth.

It seems likely that these two different enzymatic activities may have been involved in Deer's experiments, at least in the lactose-positive bacteria. Hence the changes found can be explained in terms of variations in the relative activity of each system. Actually, the data published strongly indicate that this is the case, since a different sensitivity to drying of the "lactase" as determined by oxygen consumption was found in the $L^+$ and $L^-$ strains.

REINER: I should like to add another case to the evidence mentioned by Dr. Lwoff. It is well known that dried yeast contains a strong carboxylase; and indeed an extract of dried yeast is a standard source for the preparation of the enzyme. But intact cells behave in an entirely different fashion. With *S. carlsbergensis*, strain CIAR, for example, in a large number of experiments no detectable decarboxylation of pyruvate was found under aerobic conditions, and a negligible amount under anaerobic conditions.

An obvious conclusion to draw from these results would be that pyruvate is unable to penetrate the wall of the intact cell. But if oxygen consumption is examined, it is found that pyruvate is oxidized at a high rate, and at an R.Q. close to the theoretical value of 1.2 for complete combustion.

Moreover, it is possible to demonstrate vigorous decarboxylation of pyruvate in intact cells by adding a hexose in the presence of fluoride. It is evident that the limiting factor is the supply of hydrogen donor for the reduction of acetaldehyde. The latter supposedly does not inhibit the carboxylase reaction in extracts; but this is obviously not true for intact cells. (K. Wetzel, Planta 15: 697, 1932.)

It would seem that even more caution must be exercised in interpreting experimental results in terms of selective permeability barriers than in explaining them by the absence of specific enzymes.

DELBRÜCK: E. H. Anderson has obtained further results on phage-resistant bacterial mutants with growth-factor requirements. Some of his results are of interest in connection with your discussion. All of Anderson's 27 growth-factor-requiring strains require tryptophane for growth. In all of these strains the tryptophane requirement is apparently due to the same mutational step, a mutation which causes resistance to phage T1. This mutation also causes an anomaly in the nitrogen metabolism. While the wild type can utilize ammonium as sole nitrogen source, the mutant uses ammonium only in the presence of certain amino

acids. This mutation is apparently not reversible in any of its characteristics.

One of Anderson's strains requires, in addition to tryptophane, also proline (or hydroxy-proline). This requirement appears to be coupled with a resistance to T3, T4, T7. The coupling is not obligatory, however. Some mutants with this resistance pattern do not require any growth factor, in others the growth-factor requirement may disappear by further mutation, without change of the resistance pattern. These findings are entirely parallel to those of Wollman as reported in your paper. Anderson's results have just been published (Anderson, E. H., Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B," Proc. Nat. Acad. Sci. 32: 120-128, 1946).

I would also like to make a point with respect to the use of the word "spontaneous" as applying to mutations. The word merely means that certain obvious factors which might be suspected of causing the mutations were shown to be without effect. In bacterial mutations one generally has to use a specific environment which is sharply selective for the mutant. This environment will permit the mutant to come to the foreground. It is then an obvious question to ask whether the environment not only selected but also caused the mutations.

In the case of mutations of bacteria from phage-sensitivity to phage-resistance, the selective environmental factor consists in the addition of phage, which eliminates the wild type and permits the resistant mutant to multiply. In this case it could be shown that the phage does not cause the mutations.

In your case of mutations permitting the mutants to utilize succinate, the selective medium is one containing succinate as sole carbon and energy source. Here again it seems to me an obvious question to ask whether this particular medium had an influence on the mutation rate, and as long as this has not been ruled out the designation "spontaneous" would seem improper. In view of our ignorance of the causes and mechanisms of mutations, one should keep in mind the possible occurrence of specifically induced adaptive mutations of this kind, or should exclude their occurrence in a variety of specific cases.

GLASS: Because Dr. Lwoff has deplored the lack of sexual reproduction in bacteria, as making it impossible to analyze these cases of "anaphragmic" mutations by genetic means, it may be of interest to describe briefly an analogous case from an organism with sexual reproduction, the genetics of which is very well known indeed—namely, Drosophila. Many years ago Bridges described cases of mutant genes known only by their specific suppressing action on other mutant genes. These cases are relatively numerous, but altogether too little attention has been given to them, and the nature of the action is not well known in a single instance. For

the past several years I have been studying one such case discovered to exist spontaneously in a laboratory stock. A mutant gene, erupt, results in the transformation of the central part of the eye of the fly into an organ resembling somewhat a normal antenna. But in a different chromosome of the stock there lies a suppressor of erupt, which generally completely inhibits any manifestation of erupt. By appropriate X-ray irradiation of the young embryos, however, the suppressor action is destroyed and erupt is manifested in nearly every treated individual. In experiments still unpublished, I have found that even treatment of eggs in mothers of an erupt stock lacking the suppressor gene, followed by introduction of the suppressor gene through the sperms, leads to destruction of the suppression effect in the following generation. These facts suggest that in the cytoplasm there exists a specific substance, involved in the inhibition of erupt by its suppressor, which is X-ray sensitive.

It would seem that suppressor genes must have been highly important in evolution. I have found, for example, that most stocks of Drosophila I have tested, whether or not they carry erupt, seem to carry a suppressor of erupt. The prevalence of such genes implies positive natural selection, and since they are not known to have any other function than that of their specific suppression effects, it would follow that such suppression is of great importance, presumably because of the role of the gene whose mutant effect is suppressed in development, because of its high frequency of mutation, or both. Organisms must in some way be shielded from the effects of reverse mutation of those gene changes that have had an important role in evolution. Consider, for example, a gene which is responsible for a step in the evolution of the differentiation of originally identical serially homologous organs, such as a "homeiotic" gene, like erupt, or a gene that participates in the differentiation of forelimb from hind limb in a vertebrate. The more significant the advantages of such differentiation become, the more will stability of the "switch" gene be required, and the more will effects of its mutation be nullified by the selection of other loci whose action acquires a suppressor, or "anaphragmic," action.

LWOFF: As pointed out in the discussion of anaphragmic mutations, these are of different types. And it is of course quite possible that some may be analogous to the well-known suppressor effects, if it can be shown that these are really connected with specific enzymatic changes.

# VARIATION IN LEUKEMIC CELLS OF MICE

E. C. MacDOWELL

The following report represents a still undeveloped field, which has not advanced beyond the stage of describing variation, a step that precedes genetic analysis. The general phenomena of variation associated with transplanted leukemic cells in mice have widespread parallels in transplantation of other malignant growths, but the entities primarily responsible for the phenomena are mostly unidentified and the voluminous debates on possible interpretations deal mostly with vague terms whose defense tends to raise artificial issues and to separate minds that might profitably work together. The time seems ripe for more intensive experimentation and less argument. To this end, this field is represented in the Symposium. The similarities recognizable with variations of microorganisms are highly challenging; and the brilliant assembly of principles, mechanisms, and techniques that has been presented here should provide inspiration, guides, and tools.

## THE AUTONOMY OF LEUKEMIC CELLS

The term "cell" in the title carries no implication that this is assumed to be the ultimate unit. It is used merely as the smallest unit recognizable at present. Among malignant growths, white blood cells whose aggressive multiplication is responsible for leukemia are most closely parallel to populations of protozoan parasites. These cells, existing as independent individuals, require no special supporting stroma formation on the part of the host, nor special growth of blood vessels; they do not become encysted by the host; they draw enough nutrition from their immediate environment for continued multiplication without conspicuous necrosis. By their own locomotion they may penetrate almost any tissue in any part of the body, the circulating blood providing merely a secondary conveyer. Upon transplantation into a susceptible host, they at once start their invasion, multiplying as they go. With genetically homogeneous hosts (strain C58) and standardized dosage, the process in different animals was so uniform that Potter (11) could trace its course in serial sections of hosts sacrificed at regular intervals. The channels of infiltration were identified, and the time required for a given population of cells to reach different parts of the body was plotted. Variations in the pattern and time schedule were obtained by changing the site of inoculation and by using different populations of leukemic cells. These direct observations form the primary evidence of the genetic continuity of a given leukemic population from transfer to transfer. Only a little less important for this conclusion was the demonstration of the lack of activity in the germinal centers of the hosts' lymph nodes; these nodes would become greatly enlarged by the entry of leukemic cells from the periphery, without stimulation of their own cell division. The identification of the invading leukemic cells in sections depended, first, upon their abnormal position rather than upon any specific peculiarity, as has been reported from another laboratory (1), and, second, upon the presence of immature developmental stages. The cells constituting a given lesion, say in liver, do not include any form not found in the normal process of development of the particular variety of white cells (in most cases lymphocytes). Cells may vary from $10.5\mu$ in diameter, with much highly basophilic cytoplasm and conspicuous nucleoli, all the way to $4.5\mu$, with extremely reduced cytoplasm—the mature small lymphocyte. Populations differ in the predominant cell type and in average cell size, but no population consists of cells of only one cell stage. Indeed, the whole range of cell types could be demonstrated, and, even after hundreds of successive transfers, small lymphocytes still appeared in the leukemic lesions. The normal process of differentiation is slowed down and immature cells continue to produce other immature cells, but development is not entirely suppressed. Changes in the distribution of cell sizes represent changes in the rate of differentiation, rather than a selection among intrinsically different cell types each reproducing exclusively itself. The rate of cell division is probably not abnormal for cells of the respective developmental stages. Thus leukemic cells resemble normal cells in terms of structure, development, and rate of division per stage, but an outstanding difference from normal cells is that they kill the host.

For a parasitic protozoan to kill its host is not a surprising characteristic; but, considered from the background of fixed tumors, the tendency is to deny direct toxic action and to account for death by secondary effects of the invading growth. The major difference between fixed tumors and leukemia depends upon the normal differences between the tissues involved. These differences may eventually lead to a reinterpretation of the cause of death from fixed tumors. In contrast to a transplantable tumor that is implanted in a given site and grows there (with or without metastases), leukemic cells, reacting with their environment, are free to distribute themselves through the host according to the specific capacities and predilections of the particular

population or clone. This invasion is too subtle and widespread to induce encystment. Two highly significant corollaries follow from this situation. The enormous total amount of immediate contact between invading cells and host tissues provides unparalleled opportunity for the invading cells to call forth physiological responses on the part of the host. And, accordingly, these host responses reveal subtle changes in the chemistry of the leukemic cells, and every persisting change in the properties of these cells that has selective value has immediate opportunity for expression.

Although persisting variations in the characteristics of populations of leukemic cells of the mouse are outstandingly conspicuous, almost nothing has been published on the subject beyond a few isolated observations and general statements referring to increase in "proliferative vigor" and to chronic leukemias becoming acute *(2)*. This is the first time that an attempt has been made to summarize the evidence of variation that has accumulated in this laboratory in the course of the past 18 years; many previously unpublished data are included. Much of this evidence appeared unexpectedly, and so provides the basis for a broad and systematic program, rather than its results.

It is deeply regretted that the withdrawal of Dr. J. S. Potter from the field of biology has prevented a more adequate presentation of his extensive unpublished cytological studies.

## MATERIALS AND METHODS

A population of leukemic cells, originated in a spontaneous case and perpetuated by successive transplantation in young susceptible hosts, is called a *line* and designated by a letter. In contrast, the term *strain* is reserved for an inbred pedigree of mice. Lines differ according to various criteria: frequency distribution of cell sizes, number of mitochondria, rate of cell division, metabolic rate, rate of invading and penetrating host tissues, sites particularly favored, host range, and killing time. These criteria may appear to be constant over periods of transfers, but all are subject to change. This makes it hard to estimate the extent to which line differences may represent differences between the spontaneous cases in which they arose. Changes in these criteria are partially correlated, but in most cases they are sufficiently independent to preclude the establishment of relative primacy.
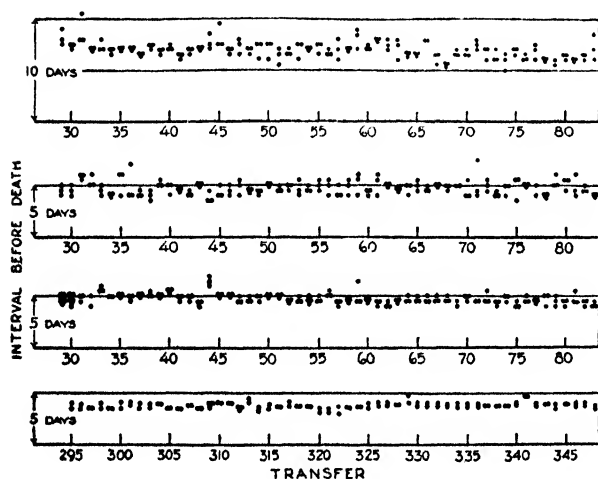
The routine procedure in transplantation is to mince into a cream a heavily infiltrated organ (usually the spleen), suspend in a proportionate amount of normal saline, mix thoroughly and continuously to maintain suspension, and inoculate intraperitoneally. The dose called standard is 0.2 c.c. of a suspension made up of one part tissue and two parts saline. Within limits, the killing time, or interval before death, is a function of the dose, so that the control of the dose is highly important. To give some idea of the degree to which this has been accomplished, Fig. 1 shows the intervals before death for individual mice in 50 successive transfers of four different lines. All the hosts, 6-8 weeks old, were taken at random from the same inbred strain (C58); the technique and dosage were the same in all cases, and yet characteristic differences in the distributions of intervals are apparent. Although the averages in the second and third lines would be similar, they show a consistent difference in variability. The first three lines were started from different pieces of tissue from the same spontaneous case; the fourth, as shown by the transfer numbers, is an older line from a different spontaneous case, but chronologically these transfers overlapped the period covered by the preceding lines, and shared in the same random selection of hosts. Another point illustrated by this figure is the seeming constancy that lines may show when a limited series of transfers is included. However, the subsequent history of each of these lines shows that none had become "fixed," and a much extended view would prove that even the seemingly stable period might be a part of a slow shift extending over many transfers.



FIG. 1. Intervals before death for four lines of transplanted leukemia, showing degree of constancy possible to attain within contemporary periods of 50 transfers (all hosts from strain C58 chosen at random). Each dot = death of one mouse; those killed as donors omitted.

### INCREASED VIRULENCE

While gradual changes in interval are far more common than abrupt ones, a sudden one will be presented first in order to show more clearly the nature of the concomitant changes in other criteria. Line M-liv originated by transferring a suspension of minced liver from a mouse ( ♀ 80524) with spontaneous lymphatic leukemia, killed 1/5/32 at the age of 335 days. The subsequent transfers were also made with liver until transfer 22, after which spleen was used. In transfers 5-17 (Fig. 2) a considerable

variability is shown, but the range and scatter is consistent. In transfer 18 all the mice died at the minimum, close to 10 days; and in transfer 19 the interval was cut in half. For the transfers before and after this break, Potter measured the longest diameter of all complete cells (800-1,000) in microscope fields of leukemic lesions. These were grouped into four arbitrary classes according to micrometer units, and the relative frequency of each class in successive transfers was plotted and connected by lines (Fig. 3). There is a shift in the distributions

reached a threshold with full effectiveness; but this adds nothing to the fact that the correlation is not at all close, and leaves entirely unexplained the very great changes in the interval before death in transfers 1-5, whereas the frequency distribution of cell size classes and the rate of cell division found in the spontaneous case originating the line (2.0% in 2546 cells counted) are virtually the same as found in transfer 12. There is a tendency to consider the first transfers of a transplanted tumor, or line of leukemia, in a separate category, and
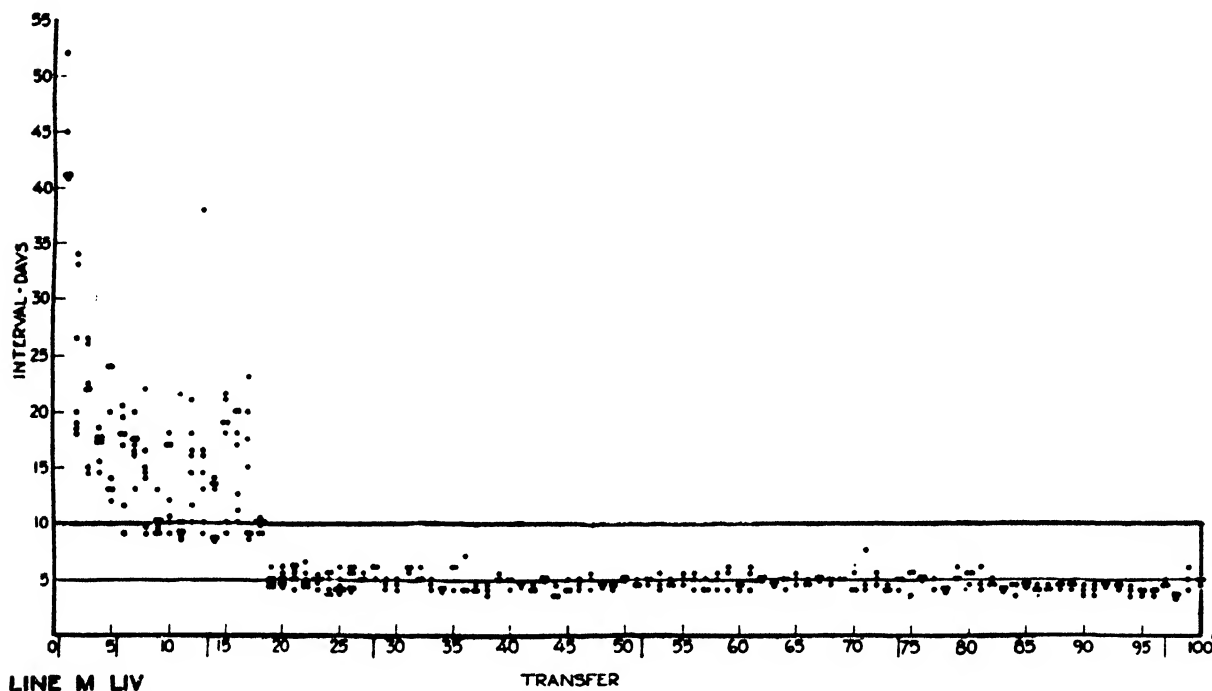


LINE M LIV        TRANSFER

FIG. 2. Line M-liv. Intervals before death for first 100 transfers. Each dot, one mouse; those killed as donors omitted.

of cell sizes, in which a strong increase in the proportion of the largest cell, class 4 $(9.0-10.5\mu)$, is balanced by a decline in class 2; class 3, with a more gentle rise, is balanced by the decline in class 1 $(4.5-5.9\mu)$. But this change preceded the break in intervals by several transfers. Another criterion of this shift in the distribution of cell size is given by measurements of metaphase plate diameters. The relative frequency of four classes in fifty metaphases per transfer is shown in Fig. 4, in which the points for the smallest size class are omitted. In accord with a widespread relationship, the rate of cell division is increased as the proportion of large cells is increased (Fig. 5).

The first inclination is to consider the change in intervals a result of the preceding increase in the proportion of large cells—that is, reduction in the rate of differentiation. This would require the assumption that the cellular changes had no effect upon the interval before death until they suddenly

brush them aside as insignificant. The picture for line M-liv tends to support this attitude. But it will shortly appear that M-liv is an extreme in a series of lines in which it becomes more and more impossible to set aside the early transfers from later ones. Rather than being a result of the cellular changes, the shortening of the interval before deaths seems more likely to be a direct effect of factors that independently and in different ways may modify the rate of cell differentiation.

The conditions found at death reflect the changes in interval and in the cells. The enlargement of organs bears a close relation to the size of the cell populations. In the case of line M-liv, the length of the spleen serves as a fair index of the total leukemic growth. (This does not mean that all organs are equally infiltrated, or that the specific organ predilections of a line are not subject to change.) During the first transfers (Fig. 5) a reduction in spleen length occurred. With the rate

of cell division and cell size not changing, the shorter intervals before death reduced the total amount of leukemic growth; but this effect was partially counteracted by a definite hastening in the process of invading the host, as shown by spleens becoming palpable earlier. Indeed, the curve for the average day spleens became palpable very closely parallels the changes in interval; although it should be especially noted that the wide variability in the time of death within transfers 5–17 had no relation in individual mice to the time spleens became palpable. After the first three

LINE M–LIV. CELL SIZE IN CLASSES 1–4
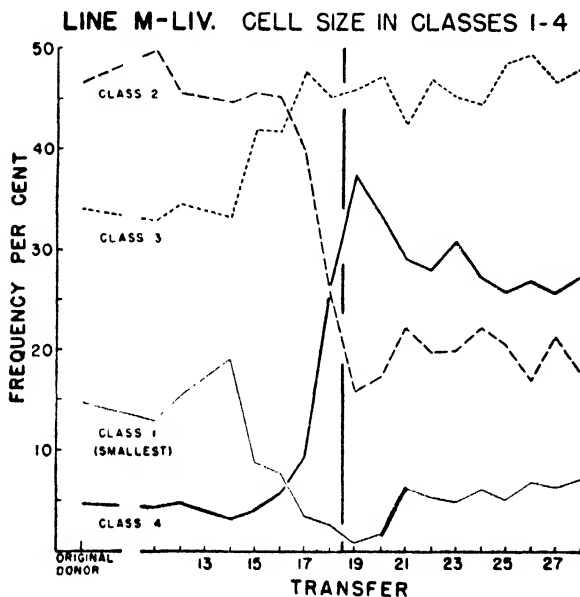
FIG. 3. Line M-liv. Relative frequencies of cell diameters grouped into four arbitrary classes, in the original donor and in transfers before and after the break between transfers 18 and 19 indicated by the vertical line; 800-1000 cells measured per transfer.

transfers, virtually all the mice inoculated in one transfer had palpable spleens on the same day. With the average interval steady and cell size and rate of cell division increasing as transfer 19 was approached, the spleens were becoming larger. But in the 19th transfer, as the intervals suddenly decreased, spleen length was abruptly reduced. This occurred in spite of the fact that spleens became palpable in half the previous time—that is, judging from the subsequent transfers, for the change in the 19th was so unexpected that first enlargement of spleens in this transfer was not observed.

The reduction in spleen length seems to be a direct consequence of the shortening of the intervals and illustrates a relationship between the total amount of leukemic infiltration and the killing time that holds very generally. The shorter the interval before death, the smaller the final volume of leukemic cells. Death is not the result of the mechanical

obstruction of a vital process, however well this may appear to provide an interpretation in spontaneous cases and clinical practice. The kinds of secondary effect usually called upon do not have time to operate in four days. Transplantation has resulted in an extremely unusual situation, but its

METAPHASE PLATE DIAMETER LINE M LIV.
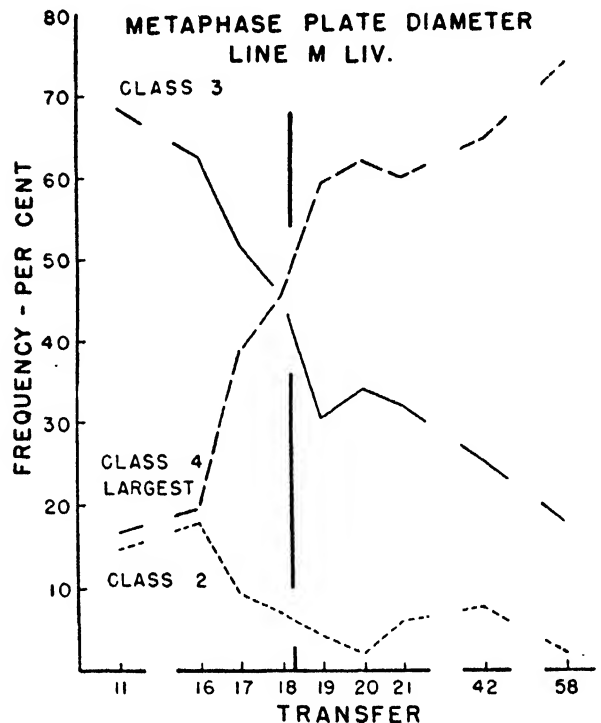
FIG. 4. Line M-liv. Relative frequencies of metaphase plate diameters grouped into four arbitrary classes, of which the smallest (No. 1) is not plotted; 50 metaphases measured per transfer.

interpretation may prove to have significance for more usual conditions. Transplantation increases the toxic action associated with leukemic cells to the point that it becomes obvious. In transfer 19 of line M-liv, the sudden increase in this toxic effect not only brought about death in half the time, with much smaller final numbers of leukemic cells, but also called out signs of shock, such as hyperemia and hemorrhagic lesions, characteristic of other highly virulent lines.

### DECREASED VIRULENCE

It is easy to understand that all changes involving more rapid penetration of hosts and more rapid cell division would have high selective advantage in the course of successive transfers. Accordingly, there is a large amount of evidence of increasing virulence, such as is illustrated above in an extreme form. But this does not say that underlying changes in the opposite direction occur any less frequently,
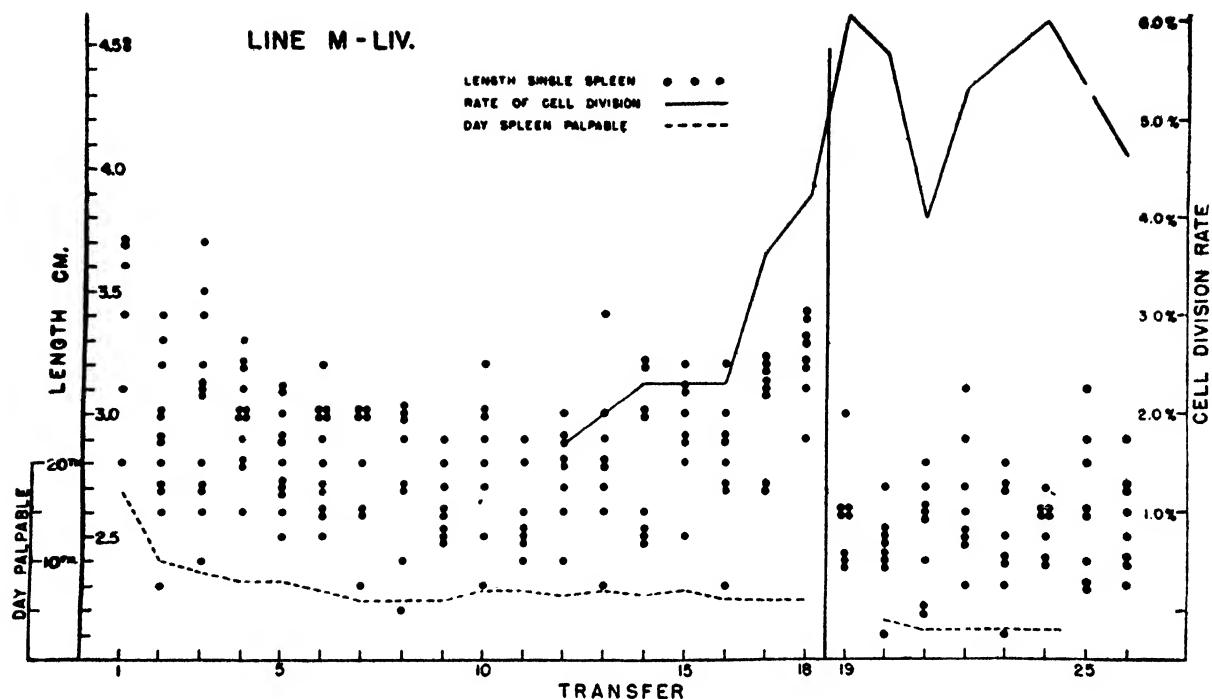
Fig. 5. Line M-liv. Rate of cell division (solid line) based on counts of 1300 (avg.) cells per transfer. Lengths of individual spleens (dots) in first 26 transfers; average day spleens palpable (broken line).
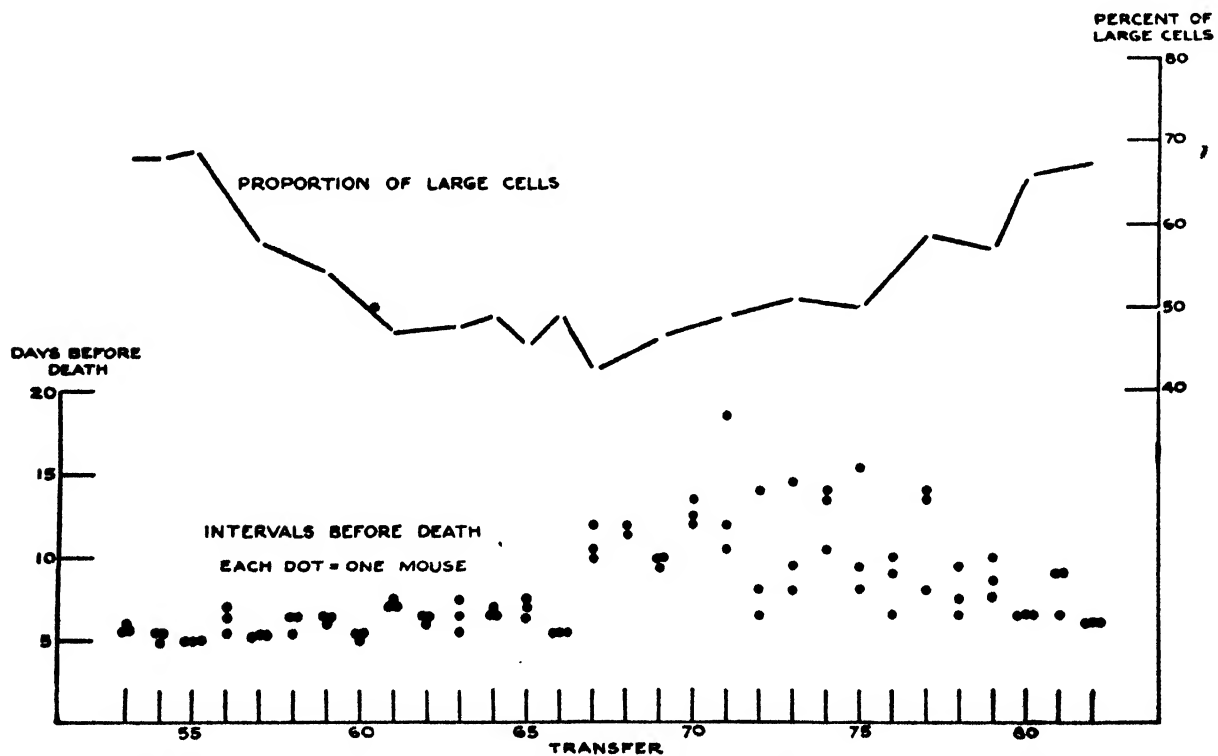


Fig. 6. Line M-kid. Intervals before death, and proportion of large cells (size classes 3 and 4), before and after a reduction in virulence in transfer 67; 400 cells per transfer measured.

or that they may not be recognized on occasion. The frequency of change is so great, and the mechanism for selection in the opposite direction is so effective, that when a change towards reduced virulence, great enough to survive at all, does occur it is soon eliminated.

One illustration of a reduction in virulence is taken from line M-kid, transfer 67 (Fig. 6); the intervals remained increased through a series of 5 transfers and then were gradually shortened again. Here again a change in the distribution of cell size classes occurred, and somewhat in advance of the abrupt lengthening of intervals, although there was a suggestion of a slight preliminary lengthening of

found during a period in the history of line I covering two years. A study was being made of the nature of these tumor masses by transplanting them into normal hosts. For this purpose the above mouse was sacrificed (age 149 days); the tumor was minced and suspended in saline and inoculated intraperitoneally into four normal hosts of the same strain. This was the ninth in a series of 27 similar tumors transplanted in this period—hence the designation, tumor IX. In every other case line-I leukemia promptly reappeared. Second transfers were made in every case, using the spleen of the donor, in order to check killing time with a controlled standard dose. In these second transfers,
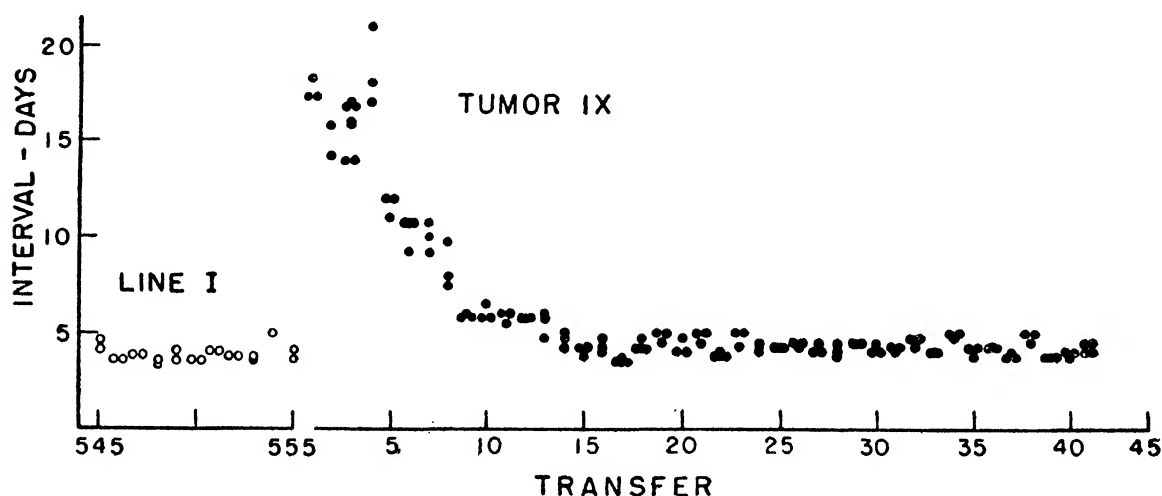


FIG. 7. Intervals before death of line I before and after conversion to a tumorous state in a host rendered resistant by a treatment with normal tissue of strain StoLi. The tumor-forming tendency faded out as the intervals returned to those characteristic of line I.

intervals. Numerous less conspicuous reverse changes of this pattern appear in the records. These grade down to cases indistinguishable from delays depending on technical factors.

A second illustration of an increase in the killing time followed by a progressive decrease extending over a series of transfers is taken from line I. This occurred in connection with a resistant state established in a naturally susceptible host (C58) by one treatment with fetal tissue from strain StoLi. The skin and viscera of fetuses at term were finely minced and suspended in 1 cc. of normal saline per fetus; 0.5 cc. of this suspension was injected intraperitoneally four days before a 100% lethal dose of cells of line I, transfer 555—a dose defined as $4^{-3}$ times standard, which according to the hemocytometer count included 1,010,000 cells and killed the untreated controls in 6½ days. After 100 days, ♂ 51719 was found to have a compact growth in the inguinal region. Under these conditions over a hundred cases of such subcutaneous growths, appearing in any part of the body, were

the mice usually died after the interval characteristic of line I at that time. Since this was not true for tumor IX, further transfers were made, continuing to use the spleen and the standard dose throughout. The intervals before death (Fig. 7), with little variation within a transfer, were gradually reduced until transfer 15, when the line-I level was reached and maintained until the series was discontinued with transfer 41.

Aside from the changes in virulence, the most conspicuous difference from line I was the reappearance of tumorous masses in all the hosts in the early transfers. These colorless, compact tumors were found in the region of the pancreas. In successive transfers these tumors were progressively smaller until in transfers 9-14 they were represented only by occasional traces. During these same transfers appeared suggestions of the inflammation of abdominal muscles characteristic of line I. At the 15th transfer, when the line-I interval before death was reached, the gross picture at death was indistinguishable from line I.

That transplanted leukemic cells may form tumor masses under certain conditions has long been recognized. Very early in the study of the first line, A, subcutaneous inoculation resulted in local tumors. Later, when the line had become more virulent, intraperitoneal and subcutaneous inoculations gave virtually the same distribution of lesions. But the tumors that appeared in the mice treated with normal tissue from strain StoLi represent more than a mechanical restriction of the invasion of leukemic cells. The tumor masses entered muscles with full replacement, instead of infiltrating between muscle fibers. The cellular constitution of the tumors themselves was highly confused and complex. Cytodebris appeared frequently, although no gross necrosis. The cellular content of the spleen, and of the lesions usually found scattered through the organs, might show different assemblages of cell types. Allowed to live, these animals very frequently developed generalized leukemia. Line I was always lymphoid, and yet myeloid differentiation was frequently noted in parts of these tumor bearers. The condition was one of great instability; the factors determining the direction of development of hemocytoblasts were fluctuating—a condition supposedly arising from a weakening of the induced resistance. From the cytological standpoint, the ease with which lymphatic leukemia indistinguishable from line I was recovered from 26 similar cases might seem a greater problem than the difficulty encountered with tumor IX. But the persistence of a tumor-forming tendency was not the only histological peculiarity of tumor IX, for the tendency to differentiate in the myeloid direction also reappeared. This was evident in sections of organs from hosts in the first four transfers, after which the organ lesions showed purely lymphoid differentiation. However, the peripheral blood continued to include myeloid types that distinguished it from the picture given by line I; the proportion of such types gradually declined from transfer to transfer until the 32nd. Before this time the proportion of hemocytoblasts had begun to increase, and after the 32nd transfer the blood picture was that of line I. The significance of this return of the characteristics of line I depends upon the high degree of individuality of the different transmission lines and more particularly upon familiarity with line I itself, which has been studied more intensively than any other.

### VARIATION IN THE RATE AND TIME OF CHANGE IN VIRULENCE

That changes in the interval before death are more conspicuous in the early transfers than later is of course very true, but this does not necessarily say any more than that the longer the killing time the more opportunity to shorten it. This is very far from saying that the early changes are proportionately much greater than later ones or that after

an initial unstable period changes no longer occur. The shorter the interval, the more difficult to detect significant changes; daily observations become too inexact, and a stage is reached at which the many extraneous factors influencing the moment of the last gasp entirely conceal the effects of intrinsic changes within the line. The interval before death approaches a limit. A line carried over 1400 transfers has never killed in less than $3\frac{1}{2}$ days. But to reach a physiological limit in terms of killing time does not preclude the continued occurrence of intrinsic line changes of the same nature, whose detection would necessarily depend upon other criteria.

There remain two points to be made from the data on changes in the interval before death: (1) the occurrence of these changes is fortuitous; (2) the absolutely large initial changes and later abrupt changes are connected by a continuous series of cases that also grade into small changes requiring long series of transfers to be recognized. These points are illustrated in Figs. 8 to 14 by portions of the histories of certain lines. Line M-spl-C (Fig. 8) shows a break similar to M-liv but not so sudden. In line M-spl-D (Fig. 9) a break of similar nature but much reduced in magnitude occurred in transfer 16; line M-mes (Fig. 10) shows an almost continuous decline, rapid at first, then much slower; in line L the decline is much less rapid. This line (Fig. 11) was derived from strain 89 and, during this part of its history, was carried in hosts of that strain. The slowness of the decline in the intervals stands in high contrast to the sudden drop in line M-liv, and yet the curve is broken up into groups of transfers, giving a highly individual pattern for the line. Line U (Fig. 12), a line originating in and carried by strain C58, has only reached its 365th transfer. This smooth continuous curve is far more common than the sudden changes that have been particularly studied. It would be hard not to believe, in the process of making the transfers, that a constant period had been reached soon after the initial decline, and the first glance at the figure may give the same impression. But a more critical study will reveal the slow, steady fall that continues throughout the series of transfers shown. To emphasize this long, slow change, which can be duplicated in several other lines, the intervals have been averaged (Fig. 13) in groups of 10 transfers up to transfer 100 and, after this, in groups of 50. Finally, line R (Fig. 14) shows wide and prolonged variability. This line was carried in hosts of strain Bagg albino (Balb), another highly inbred strain unrelated to C58. For a few transfers the intervals were closely grouped, then appeared a brief period (transfers 8-10) with great variability, which reappeared in transfer 22 and persisted for nearly 60 transfers. During this time the minimal intervals throughout tended to decrease at a uniform rate. Eventually the wide variability was
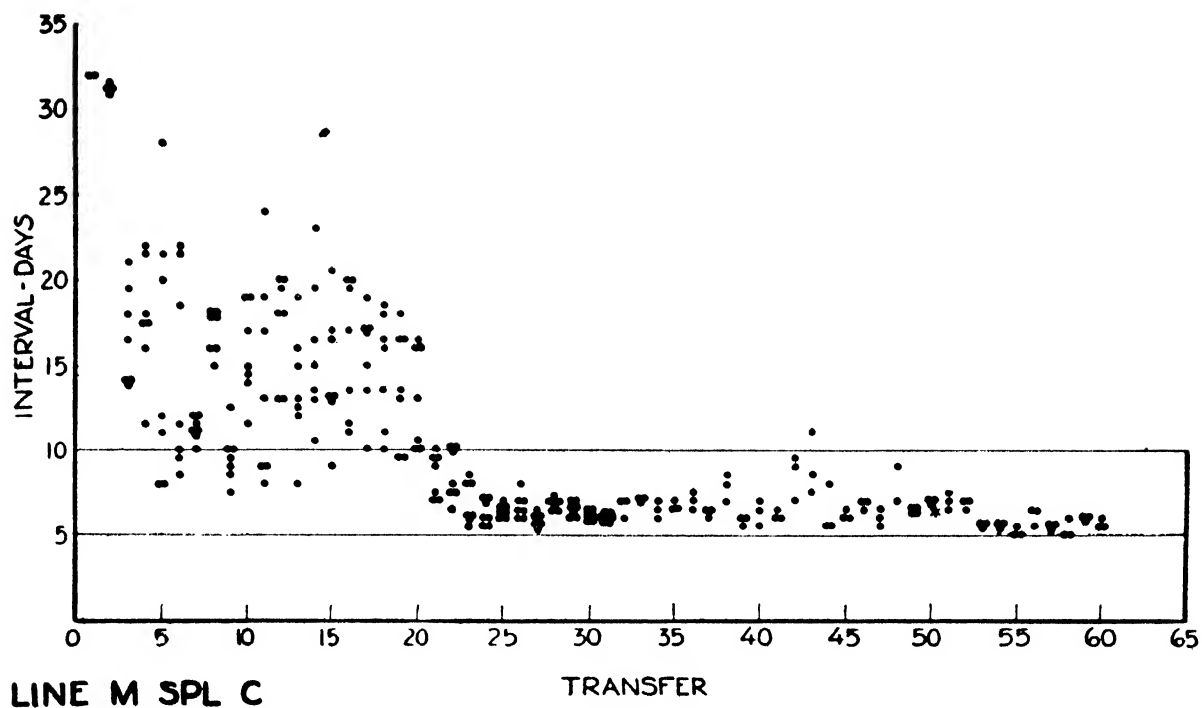
## LINE M SPL C

FIGS. 8-14 show variations in the time and rate of changes in intervals before death in
different lines. FIG. 8, Line M-spl-C.
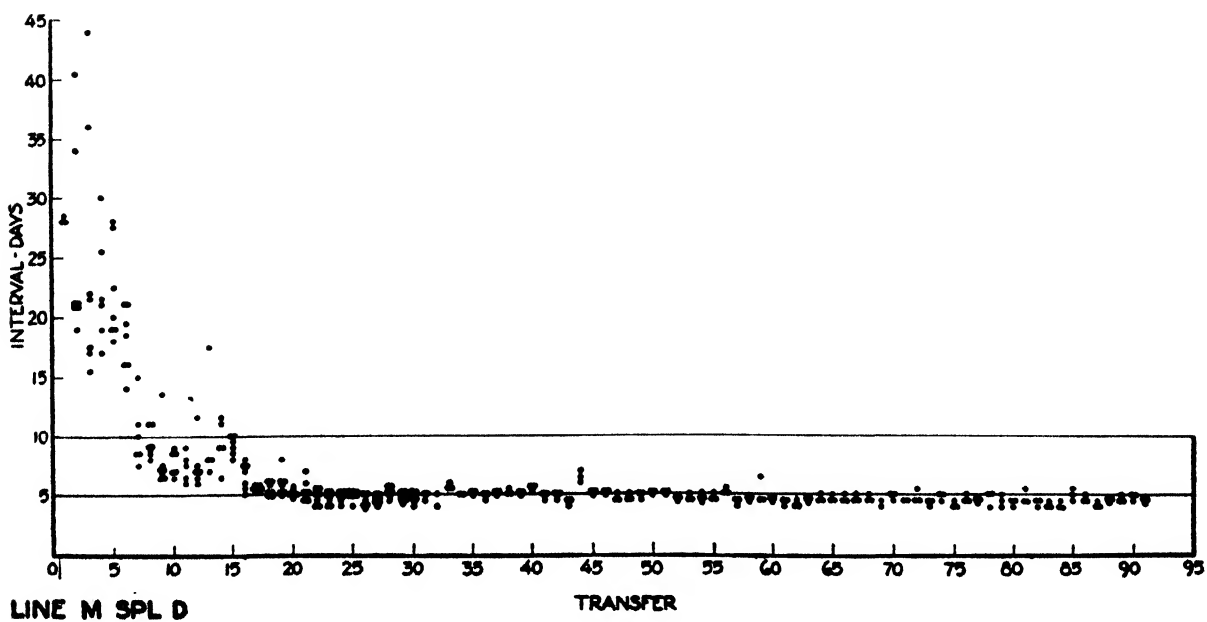


## LINE M SPL D

FIG. 9. Line M-spl-D. Intervals before death.
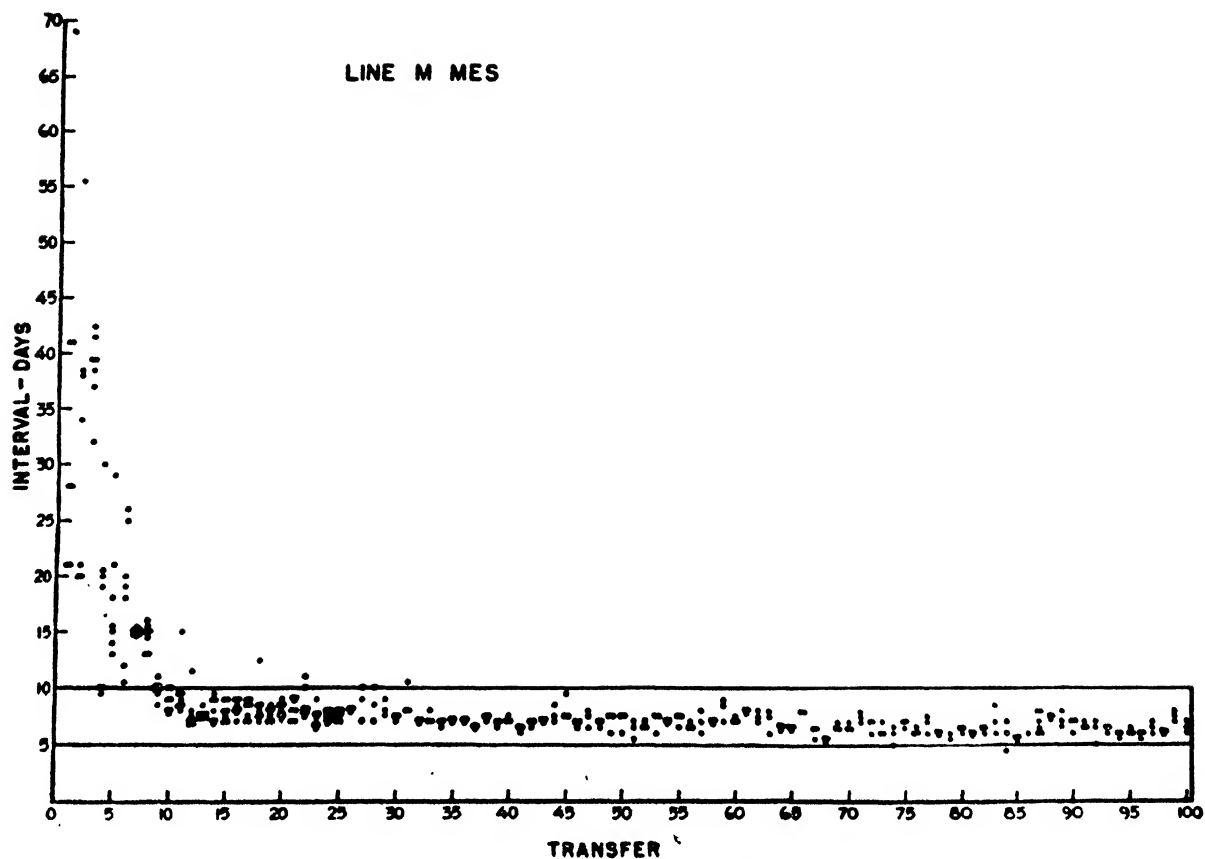
FIG. 10. Line M-mes. Intervals before death.
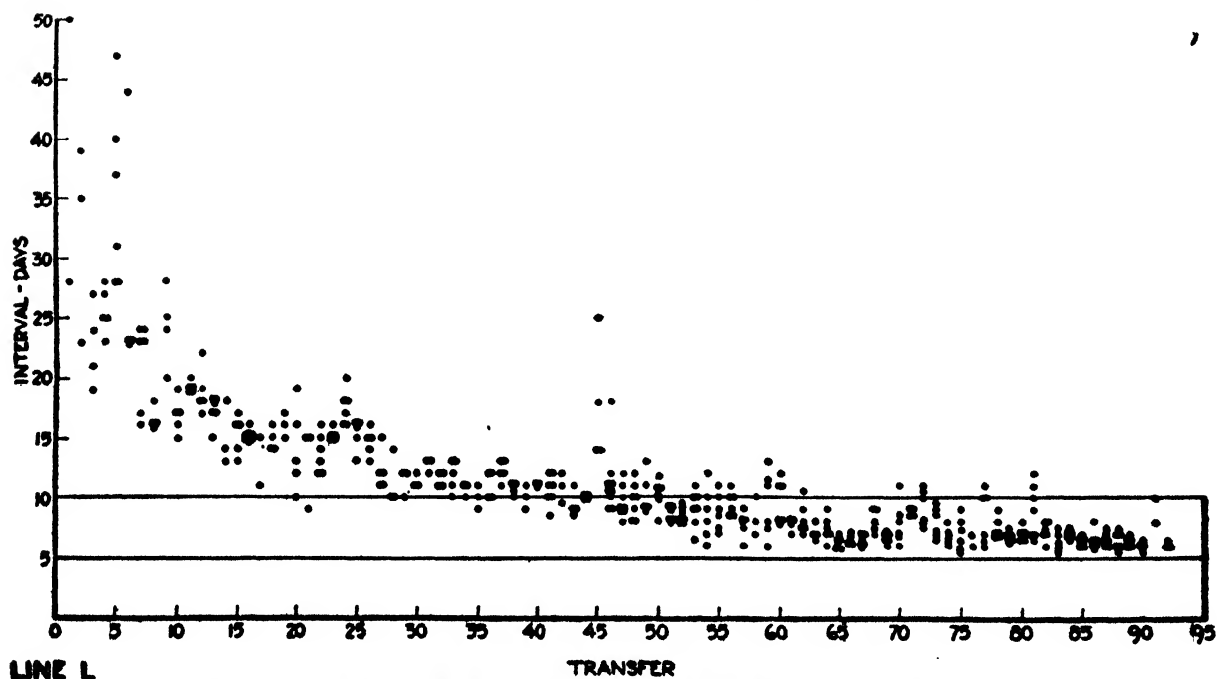


FIG. 11. Line L. Intervals before death; this line originated in and carried by strain 89.

eliminated. A closely similar history of variability of even greater range was that of line A, a C58 line.

Considering the broad range of situations presented by different lines of transplanted leukemia and in different periods of the same line, the length of the interval before death under controlled dosage is generally associated with the proportion of the largest cells, the rate of cell division, the number of mitochondria, and the metabolic rates, especially anaerobic glycolysis. The cellular changes, as in
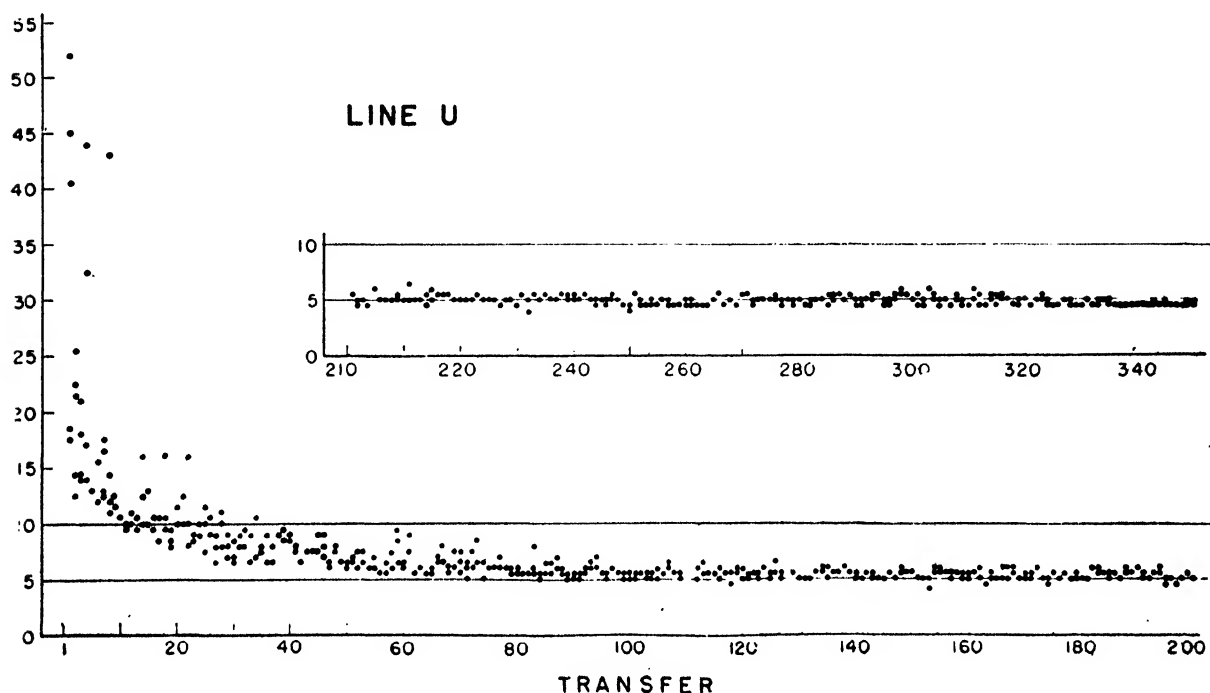


Fig. 12. Line U. Intervals before death. In this figure, for greater accuracy of the silhouette, no more than one mouse dying at the same time is plotted. These are indicated in other figures by groups of overlapping dots.

TABLE 1. The Effects of Many Transfers, Dosage, and Strain of Host on Conditions at Death.
Data from Experiments of Dr. G. Gasić

| Source of Leukemic Cells | Transfer | Dose | Number of Mice | Interval (Av.) (Days-Hours) | Organ Weights, mgms. | | | | | Lesions | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Liver | Spleen | Thymus | Adrenal | Pancreas | Hemorrhagic | Hyperemic | Edema |
| Own Hosts—Strain C58 | | | | | | | | | | | | |
| Uninoculated controls | | | 54 | | 1168 | 91 | 48.7 | 4.9 | 184.6 | | | |
| Line I | 1420+ | Std. | 12 | 4$^3$ | 1470 | 490 | 22.8 | 8.9 | 116.5 | ++ | +++ | + |
| Line I | 1420+ | 4$^{-7*}$ | 10 | 9$^{18}$ | 1929 | 766 | 19.9 | 8.2 | 112.1 | + | + | + |
| Spont. | 1 | Std. | 10 | 15$^{10}$ | 2270 | 1004 | 58.5 | 4.5 | 132.0 | ± | + | + |
| Spont. | 1 | Std. | 8 | 34$^5$ | 2870 | 1008 | 52.2 | 6.0 | 165.0 | + | + | + |
| Spont. | 1 | Std. | 10 | 46$^{21}$ | 4550 | 1715 | 60.5 | 5.6 | 138.7 | ± | + | + |
| Foreign Hosts—Strain Balb | | | | | | | | | | | | |
| Untreated controls | | | 8 | | 1280 | 86 | 63.3 | 5.8 | 154.0 | | | |
| Line I | 1419+ | Std. | 10 | 4$^5$ | 1210 | 126 | 16.3 | 6.3 | 127.3 | +++ | ++ | + |
| Line I | 1418 | 4$^{-4*}$ | 5 | 8$^6$ | 1140 | 98 | 11.1 | 5.1 | 76.0 | ± | +++ | Note** |

* Times standard.      ** Large amounts of pleural fluid.

the case of transplanted tumors, are in the direction of decreased differentiation. But when individual lines are compared, it becomes evident that the various criteria are not directly correlated. For example, lines with the same average interval before death may differ in metabolic rates, and the order
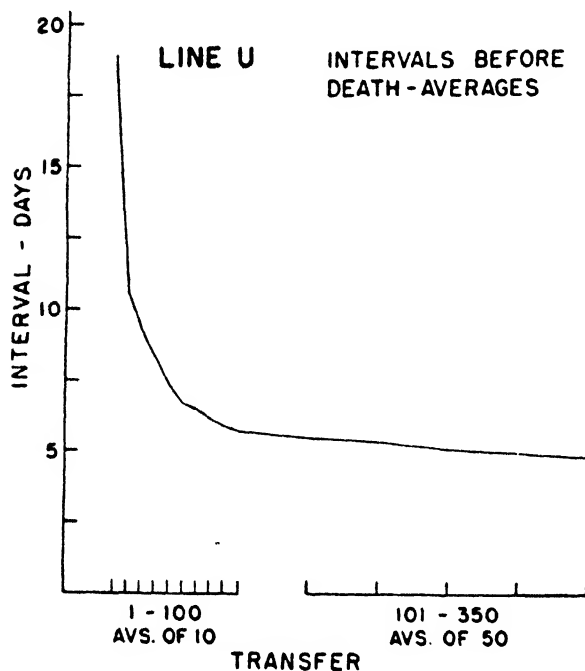


FIG. 13. Line U. Intervals before death averaged.

of a group of lines according to their intervals may not be the same as the order of their metabolic rates (15). But the association of very short intervals with signs of toxic action and small leukemic lesions appears to be direct and dependent.

Dr. Gabriel Gasić, who has been studying these shock phenomena, graciously offers extracts from his extensive observations to show certain reactions. His meticulous autopsies have included weights of various organs and glands as well as estimated grades of their hyperemic, hemorrhagic, and other lesions. The first section of Table 1 compares the action of a line killing in 4 days, after many transfers (line I, 1420-1428), with the first transfer from three spontaneous cases (killing averages respectively 15, 34, and 46 days).

The relative size of the terminal leukemic populations, as indicated by the increase in weight of the liver and spleen over the controls, was considerably less with line I than with the three first transfers. On the other hand the signs of toxic action (involution of the thymus and pancreas and vascular lesions) were more conspicuous with line I.

CHANGES IN HOST RANGE

The changes already described have an intimate connection with increases in the range of hosts susceptible to a given line. Host range is a common criterion for detecting changes in organisms and in transplantable tumors. Innumerable parallels with the following results will of course be evident, but it is their deviations that deserve special consideration. This applies particularly to the deviations of these results from results with transplantable tumors.

In the classical pattern, line I (transfers 23-100) was resisted by all hosts in the unrelated and highly inbred strain StoLi (Storrs-Little). Using for the test dose line-I cells that had been carried exclusively by hosts of its strain of origin (C58), all $F_1$ hybrids between strains StoLi and C58 were susceptible and half of the backcross (BC) of $F_1$ to StoLi were susceptible. Results from parallel experiments with transplantable mouse tumors have been interpreted by Mendelian ratios calling for various numbers of genes and occasionally only one, as above. Although these interpretations fitted the facts and were statistically supported by probable errors, it was still possible to question the validity of these genetic conclusions (4), which were based on an all-or-none classification of a complex phenomenon subject to the influence of numerous nongenetic variables. Probable errors could help decide between different theoretical genetic formulas, but such statistical calculations could not demonstrate that the results were directly determined by the segregation of any gene or genes; the crucial evidence that the phenotypic ratios actually represented genetic differences was lacking.

The fact that such evidence for the present case has been obtained has importance for the interpretation of the line changes that later appeared, and would seem to have theoretical implications of wider application. The conventional method of breeding tests was used to demonstrate that the above one-to-one ratio for backcross mice susceptible or resistant to line I was in fact a genetic ratio (9). Fifty backcross males were mated with inbred StoLi females, and the proportion of susceptible young in each of the 50 families produced by these matings was determined by inoculation. Thus the BC animals could be described by an unlimited percentage scale instead of an all-or-none reaction, and the nongenetic variables that might be the deciding factors in individual cases were counterbalanced by the random sampling. Twenty-five of these families gave ratios evenly distributed about 50%; the other twenty-five families were almost entirely resistant. The total for the two groups of families gave 47.7% susceptible among 618 and 0.9% among 705 (Fig. 15). After the BC fathers had completed their breeding, although far beyond the standard age for making such tests, they were inoculated with line I. None of the fathers of the resistant families

died, against 17 of the fathers of the 50% families. Their age may account for the unusually low proportion of susceptible BC mice. The slight deficiency of susceptible mice of standard age that persists in each backcross total, compared with the invariable susceptibility of the $F_1$ hybrids, indicates

host strains, and (2) that another line of leukemia (M-spl-B) inoculated into BC mice of the same cross, but raised after the change in line I was discovered, gave a 30% preponderance of resistant mice while line I was giving a 30% preponderance of susceptible mice. Fig. 16, which summarizes
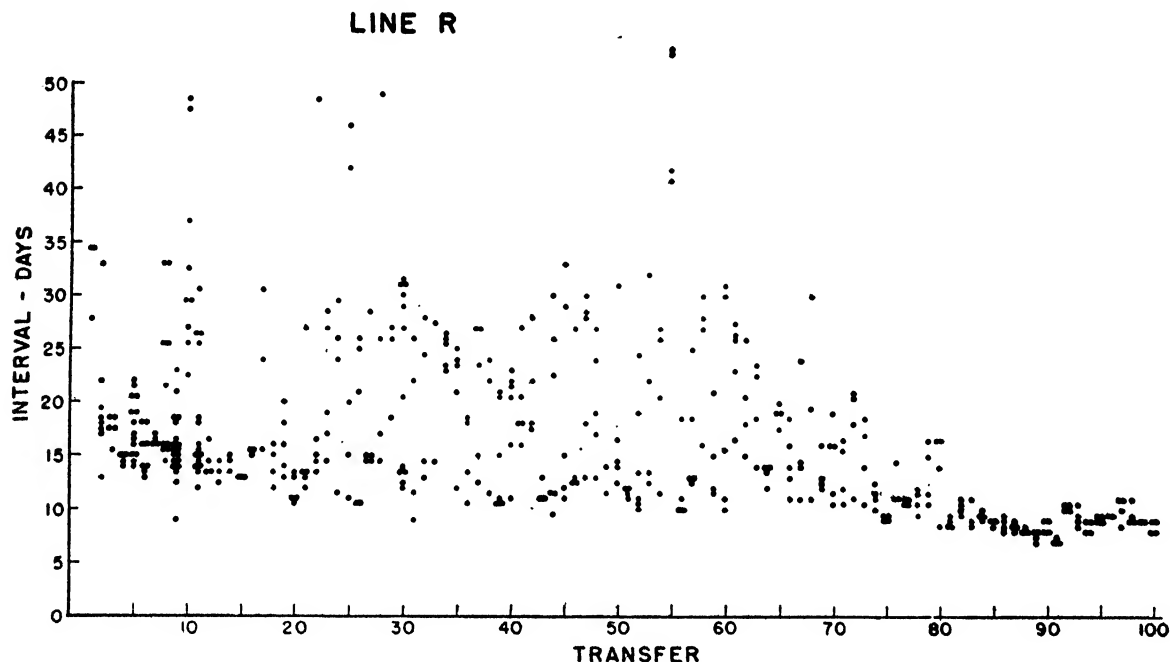
## LINE R



FIG. 14. Line R. Intervals before death; this line originated in and carried by Balb.

a minor influence of other segregating genes. Further evidence of the occasional survival of a heterozygote was provided by breeding tests of 40 backcross survivors. These were bred together in shifting combinations and their offspring inoculated (87 matings, 932 young tested). One of these second backcross survivors was found to be heterozygous for susceptibility.

Strains C58 and StoLi differed in one particular gene pair, whose segregation in the backcross was directly revealed by the proportion of mice susceptible to line I as constituted between the 23rd and 100th transfers. This would seem to clarify the issue decisively, at least for the present case.

A subsequent test of backcross mice of the same constitution made with line I, 143, gave more than three-quarters susceptible. This indicated that not only all of the heterozygotes were dying, but also many of the homozygous recessives. Accordingly a test of inbred StoLi mice was made, and 8 out of 18 died; still later (transfer 197) line I was carried by StoLi hosts for a series of 14 transfers. That this was the result of a change in line I rather than in strain StoLi is indicated by the facts (1) that changes of the same nature were indicated by the results of inoculating line I into two other inbred

these results with line I over the period under consideration, shows that the proportion of susceptibles in inbred strain dbr jumped from 20% to 80% at some time in the neighborhood of the 110th transfer, but strain 89, which gave a much higher proportion when first tested, did not show an impressive increase in the proportion of susceptibles until 30 transfers later. It is entirely understandable that two foreign strains should not only exhibit different degrees of susceptibility but also respond differently to changes in line I. The tests of line M-spl-B with BC hosts were made very early in this line's history (Fig. 17). Its intervals before death in C58 hosts were considerably longer than those of line I at the same chronological time, and one C58 mouse had survived. It seems difficult to avoid the conclusion that these two lines originating in C58 and carried by C58 hosts differed in their host ranges and that these differences were related to the time required to kill hosts of their own strain, and, further, that the differences between these strains closely resemble the differences found at different periods within line I. This suggests that before the genetic study was started (transfer 23), line I may also have given less than 50% susceptible in the BC. This was the case in two other lines

(E and H, Figs. 18 and 19), which gave far less than 50% susceptible when first tested in BC hosts of this same cross, while later transfers, with increased virulence, approximated 50%.

The dependence of increased host range upon the line of leukemia rather than upon changes in host strain is well shown by C58 hosts when tested with the foreign line L at different periods in its history. As the intervals before death decreased in hosts of its own strain (Fig. 11) the proportion of C58 hosts in which leukemia developed increased
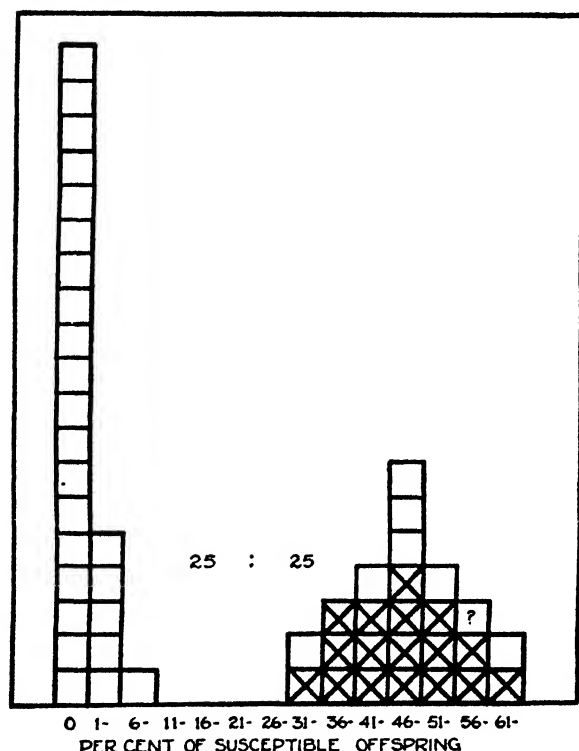


FIG. 15. Inheritance of susceptibility to line I, transfers 23-100. Fifty backcross mice (C58 × StoLi) × StoLi, were mated again to StoLi, and the proportion succumbing to inoculations in each family shown by a square; after breeding, the backcross parents themselves were inoculated.

from 1% to 95% and, eventually, 100%; and after this, for convenience, line L was regularly carried in strain C58. With mice of strain C58 being taken at random, from any branch of the current pedigree, as hosts for numerous lines contemporaneously with these tests with line L, and with each line exhibiting its own characteristics and consistent history of change, the assumption of random mutations in the host strain to account for the progressive changes in the proportion of C58 mice killed by line L, in the absence of any possible selective mechanism, becomes untenable.

Continuing with line I, the intervals before death of C58 hosts (Fig. 21) indicate that changes were

occurring. The intervals for the second forty transfers shown in the figure are slightly shorter than in the first forty, and after transfer 100 there is further very gradual but unquestionable shortening. More accurate observations might well have revealed that the gradual change extended over the entire period. Line I was becoming increasingly pathogenic; hosts of strain C58 were dying sooner and sooner, and larger proportions of hosts from other strains were dying, but it is not possible to point out any direct temporal correlation between the changes shown by different host strains. With strains differing in the threshold between survival and death, the same intrinsic changes in line I that are effectively continuous, as expressed in C58 hosts, may be responsible for seemingly discontinuous and nonsimultaneous effects in other strains.

That line changes may be expressed by changes in the proportion of susceptibles from highly inbred strains (line I in dbr and 89; line L in C58) indicates that direct genetic interpretations of the proportions of susceptible BC hybrids and of changes in these proportions would have little significance without further experimental evidence. The assumption that qualitatively different line changes are specifically related to the genes of the host, whether these be of an inbred strain or segregating hybrids, is as unnecessary as the assumption that discontinuity according to some one criterion is evidence of gene mutation within the leukemic cells.

From the standpoint of the investigator, it was just a fortunate accident that sufficient evidence to demonstrate a gene for resistance was obtained before line I had accumulated enough virulence to kill mice homozygous for this gene. After this point had been reached, line I still gave distinguishable results in different host strains. Not all the BC mice were killed, and the 100% susceptibility shown on the chart for StoLi gave way in subsequent tests to some recoveries. Line I had lost its susceptibility to the action of a particular gene, but it would be incorrect to say that it had thereby lost all its specificity and that it would thereafter grow equally well in hosts of all strains. Such, however, was the interpretation offered for a change in a transplantable tumor, dbrCX (*13*), that had been giving a proportion of susceptible $F_2$ hosts explained by 6 theoretical genes. The change increased the rate of tumor enlargement approximately 22 times. Considerably more data than are given would be necessary to demonstrate that a tumor had lost "all its specificity" and that it had become independent of all kinds of host-strain differences. Indeed, even among the data published appear two unexplained $F_2$ mice that were not susceptible to this changed tumor. Other transplanted tumors also have shown changes in the proportion of susceptible hosts in a segregating generation, and these changes have always reduced the number of genes required for

the interpretation (3). But these changes were small enough to leave some theoretical genes to account for the results. This gave the impression that all susceptibility depended upon genes and carried the implication that the changes in the

For one period of line-I leukemia, a gene of the host turned the balance between resistance and susceptibility. It seems highly probable that some of the ratios from tumor implantation were also dependent upon one or more segregating host genes,
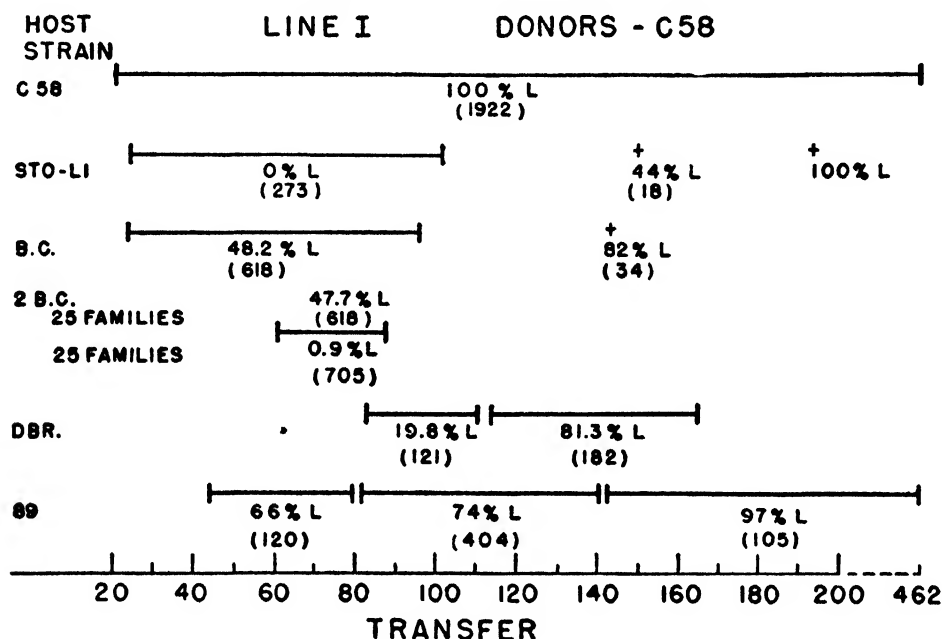


FIG. 16. Percentage of deaths from line-I leukemia at different periods, in hosts of various genetic constitutions; total inoculated in brackets; BC = (C58 × StoLi) × StoLi; 2 BC = BC × StoLi; totals for the two groups of families shown in Fig. 15.

tumors themselves must in some way be geared with genes, if not immediately dependent upon them. When the potency of a tumor had increased to the point that only one gene was necessary to interpret



FIG. 17. Line M-spl-B inoculated into BC and $F_2$ (same cross, see Fig. 6) after the change in line I was discovered.

the results, the case was heralded as a final confirmation of the whole theory. But this general theory did not call for more than 50% of the backcross or more than 75% of an $F_2$ to be susceptible; and so when such results were obtained, they were brushed aside into a special category.

as they have been interpreted. But the conclusion that specific host genes turn the balance between susceptibility and resistance to a tumor in one condition, does not justify the generalization they do so for all changes that may appear in this tumor. If susceptibility, before and after all changes in tumors or leukemia, is not necessarily directly dependent upon certain specific genes of the hosts, (1) it becomes a matter of chance that a tumor of a certain make-up is resisted by a particular host gene or genes, (2) it is no longer necessary to suppose that the amount, or quality, of change in a tumor has any connection with its genes, (3) it causes no confusion to find that neoplasms may acquire such potency that the proportion of susceptible hosts in an $F_2$ generation exceeds that expected from the activity of a single gene.

### MANIFESTATIONS OF TOXICITY

A line of leukemia that has developed sufficient pathogenicity to kill hosts of strains other than its own does not necessarily lead to death by the same course of events in different host strains. Strain differences in the size of the leukemic lesions and in the intensity of the signs of toxicity are obvious. Sections of mice from strains StoLi, Sh, Sil, Daab, Balb,

Balb ab that had died after inoculation with line I were described by Potter as "recovery," corresponding to some stage in the process of leukemic cell destruction reported by Potter and Findley (*10*) in C58 hosts rendered resistant by experimental pro-

HOST LINE E. DONORS STRAIN C58
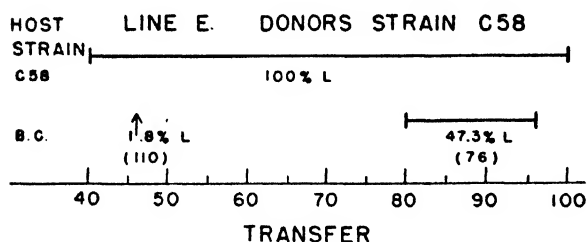


Fig. 18. Line E in backcross of same cross, showing a change from a very few to practically half susceptible.

cedures. The current observations of Dr. Gasić on liver and spleen weight give a quantitative measure of the amount of this suppression of line I in Balb hosts compared with C58 hosts (Table 1). The standard dose of line I was killing hosts of these two strains in approximately the same time. In C58 the

HOST LINE H DONORS - C58
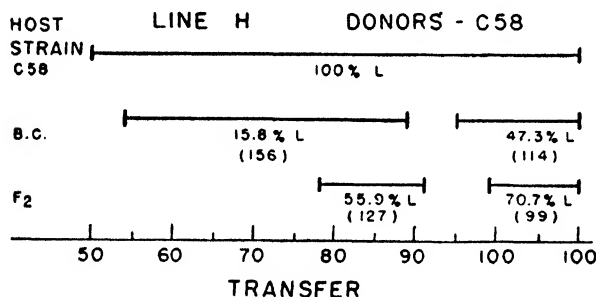


Fig. 19. Line H in BC and F₁ of same cross, showing an increase in proportion of susceptibles.

leukemic livers, at death, were 25% heavier than the uninoculated controls; in Balb they were 5% lighter than the uninoculated Balb controls. The leukemic spleens weighed 5.4 times the normals in C58 and 1.5 times the normals in Balb. The involution of the thymus was more advanced in Balb and

the hemorrhagic lesions more extensive, although hyperemia was less intense. By diluting the dose of cells, the terminal picture may be modified. This fact has long been recognized in this laboratory and is confirmed by extensive observations. Within limits, dilution of the dose not only lengthens the interval before death, but, in fully susceptible hosts, increases the size of the final leukemic populations and decreases the signs of shock. Dr. Gasić gives quantitative evidence of this phenomenon, and adds the highly significant observation that a dilute dose in a foreign host varies the picture in a different direction. In C58 hosts (Table 1) a dose of line I, transfer 1428, diluted to $4^{-7}$ times standard (approximately 4000 cells), more than doubled the interval before death, but still killed all the mice. (Formerly $4^{-4}$ times standard was the lowest 100% lethal dose.) The increase in liver weight rose from 25% to 65% of normal and the spleen weight from 5.4 to 8.5 times normal. The vascular lesions were strongly reduced but the gland weights not much changed. In Balb hosts, the dilution of $4^{-4}$ times standard was the lowest lethal dose of line I at this time; this gave the average interval before death within a day of that from $4^{-7}$ in C58 hosts. But the livers and spleens, in contrast to C58, were definitely lighter in weight than with the standard dose, the livers averaging 11% less than normal. The involution of thymus and pancreas was increased, and, although hemorrhagic lesions were reduced, the hyperemia was increased and large amounts of pleural fluid appeared in every mouse. Pneumonia was absent.

The above differences between host strains with the standard dose and their differential responses to dilute doses are important for their bearings on the interpretation of changes within a line in terms of host range. They are also important in showing that resistance to the proliferation of a line is not identical with resistance to its lethal action. Under conditions that permit the free proliferation of leukemic cells, the full effects of the toxic action are masked. With line I in C58 a depressing effect upon the thymus is demonstrable, but in heavily infiltrated organs, such as liver, this effect is not recognizable. In Balb hosts, at least in the terminal stage, the

HOST LINE L DONORS STRAIN 89*



* LINE CARRIED IN C58 HOSTS AFTER TRANSFER 792
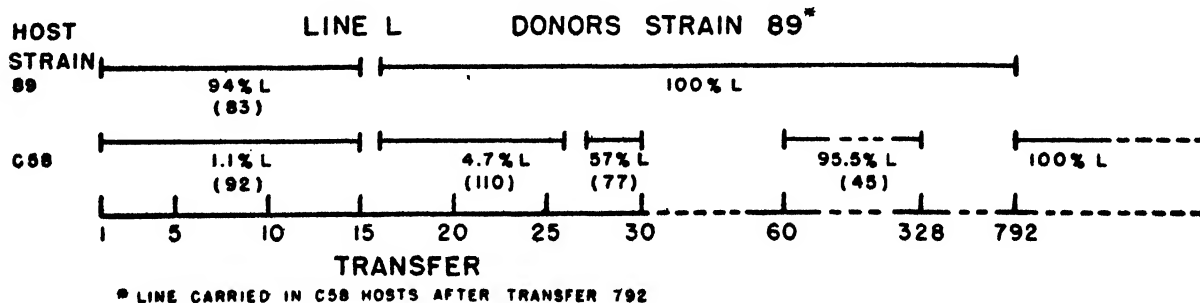
Fig. 20. Changes in line L (originated in strain 89) indicated by proportion of susceptible C58 hosts.

leukemic lesions of the liver are so reduced that a depressing effect upon the normal tissue of the liver is evident.

The first indication of the deleterious action of leukemic inoculations, separated from the immediate presence of the invading cells, appeared in the metabolic studies of Victor and Potter (*14*), who discovered that the lymph nodes of C58 hosts inoculated with lines A or I showed increasingly depressed metabolic rates until the invading cells entered the tissue and raised the metabolism above normal. In these experiments lymph nodes were

## LINE DIFFERENCES AND CHANGES IN TERMS OF INDUCED RESISTANCE

### Resistance Induced by Normal Tissue

A single treatment with normal tissue (fetal skin or viscera, adult liver) from strain StoLi creates a condition in C58 hosts that enables them to resist an otherwise 100% lethal dose of line I, $4^{-8}$ times standard. Normal tissue from strain C58 has no effect. It is interesting that the intraperitoneal injection of 0.1 cc. of minced StoLi tissue will induce resistance, when the $F_1$ hybrids from a StoLi mother
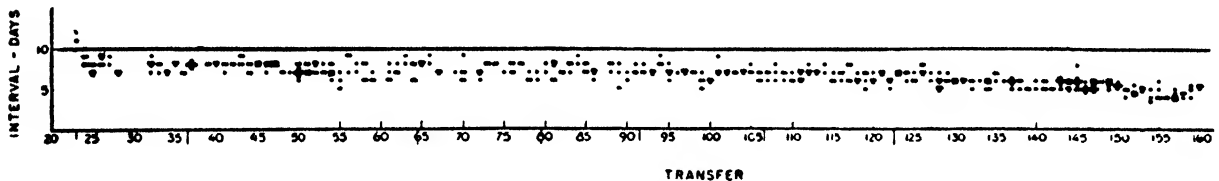


FIG. 21. Line I. Intervals before death. Transfers 23-100 were resisted by all hybrids homozygous for one particular StoLi gene.

surgically removed at intervals of 24 hours preceding and following inoculation. Although previous studies of Potter on the rates and paths of infiltration had given close estimates of the time required for the invading cells to reach a given lymph node, the presence or absence of leukemic cells in every node studied was independently checked by sections. The most important metabolic changes were in the rate of anaerobic glycolysis. Leukemic cells themselves exhibit a high rate, but they are responsible for a depressing effect upon host tissues. The question remains, how is this metabolic effect related to the other indications of toxic action and to the cause of death?

Still further separation of pathogenic activity from growing leukemic cells has been accomplished in experiments with heat and centrifugation. Subjecting cells of line I (transfers 829-879) in standard saline suspension to 46° C. for ten minutes caused only very subtle changes in the appearance of the cells, but prevented their proliferation in C58 hosts. However, all such animals showed a sickness that was usually followed by a slow recovery. The same result followed inoculation of C58 hosts with the "small particles" separated from line I by Claude using differential centrifugation (*6*). An important control in these experiments was provided by another line. At that time line S was much less virulent than line I, according to the intervals before death (8-10 days, compared with 4 days) and also according to the terminal picture. Using the same techniques, line S in C58 hosts gave no sickness either after the heat treatment or after the differential centrifugation.

and C58 father are invariably susceptible, in spite of the presence of a full set of StoLi chromosomes in every cell, to say nothing of the StoLi egg cytoplasm and the uterine contact and nursing. When the test dose of leukemic cells was given at the same time or a day after the normal tissue, all the mice died at the same time as the controls; but when it was given three days afterwards, well-developed resistance was evident in all mice. Before transfer 674 of line I, serial section of the organs of such resistant mice, sacrificed at daily intervals after the test dose, gave no indication of any leukemic cells up to the time of the controls' death. The final outcome was either (1) indefinite survival, (2) delayed leukemia, with death far beyond that of the controls, (3) tumors, with generalized leukemia usually appearing before death. Reference to these tumors has been made above.

After transfer 674, line I, in mice treated with normal StoLi tissue, gave different results. In serial sections of daily series the invading cells could now be distinguished (*7*). Their rate of division was less than that in the controls without normal-tissue treatment, and the lesions they formed were smaller, but when the controls were approaching death the process of cell destruction *in situ* began and soon wiped out the leukemic population. The final outcome was uniform; all survived indefinitely. The cases of delayed leukemia and the tumors that formerly appeared were no longer found.

Other lines against which treatment of C58 hosts with StoLi tissue has been found to induce resistance are M-liv, M-kid, A, and L. Lines not resisted by this treatment are S, T, U, and first transfers from

14 spontaneous cases. Line R, carried by Balb hosts, was not resisted when hosts of this strain were treated with StoLi tissue; rather, the enlargement of the spleens was hastened and the interval before death was somewhat shortened.

When it was found that the relatively avirulent, slow invading cells taken directly from a spontaneous case were not resisted, while the highly virulent line-I cells were (8), the question naturally arose whether high virulence was necessary. Accordingly, three new lines were started which were to be tested periodically as their virulence increased. Each test included 10 males treated with StoLi liver three days before inoculation with the lethal dose $4^{-3}$ times standard and 10 untreated control males from the same litters and in equal numbers. All were inoculated in alternate groups of five with the same suspension of leukemic cells.

Line S has been carried for 470 transfers and tested at 33 different times; line T was tested 5 times before the 71st transfer, when it was accidentally lost; line U has reached its 365th transfer and has been tested 18 times. Lines S and U are killing, with the standard dose, in 4½ days, which is as fast as line I was killing when it was first used in tests of normal-tissue resistance; and yet neither of these lines, nor line T, has been resisted in any way comparable to line I. The intervals before death throughout all the tests were indistinguishable from the controls. The only indication of a possible effect of the treatment was in the survival of 8 of the 180 treated mice tested with line U. Of these 8, 6 were in the experiment testing transfer 158; in this experiment one of the controls survived after the dose of $4^{-3}$ times standard, which in all other experiments was 100% lethal. The 7th test survivor was in the fourth test following (transfer 242), and the 8th in the third test following that (transfer 342). These survivors also survived three or more reinoculations with larger doses of line U. Livers from such resistant mice were finally used to test for the so-called passive transfer of this resistance. Following the routine procedure used for the passive transfer of resistance induced to line I (12, 5), the intraperitoneal injection of 5 cc. of a saline suspension including 0.1 cc. of the minced liver was followed at once by the lethal dose of line U, $4^{-3}$ times standard. Liver donors that had survived doses of line U, $4^{-3}$, $4^{-1}$, standard, standard, standard, were used in two experiments. A total of 17 mice out of 20 survived. In one of these experiments 10 additional mice treated with the immunized liver were inoculated with line S, $4^{-3}$ times standard; all of these died at the same time as their 10 litter-mate controls inoculated with the same dose of the same suspension of line-S cells; death occurred in 8 to 10 days, with average interval for tests 9.07, for controls 8.95. These numbers are not impressively large, but the degree of control of the biological and experimental variables that has been attained gives these results considerable significance. It may be noted, incidentally, that such line specificity is demonstrable with passively transferred resistance, even in cases in which the mice actively immunized by one line will resist the second.

Whether these few StoLi-treated survivors of line U and their ability to transmit passively the resistance that was built up in them are indications of change towards susceptibility to the condition induced in hosts by treatment with StoLi tissue, only the future course of events can tell. Broadly, those lines against which treatment with StoLi tissue is effective are those with the capacity to induce active resistance to themselves. In the case of line I, this relation is very close, for the mice that have been treated with StoLi tissue and tested with the dose $4^{-3}$ times standard are as able to resist repeated standard doses as mice that have been rendered resistant by a course of very dilute doses of line I. But it is important to bear in mind that this actively induced resistant state, although transmissible passively, is not able to prevent extensive initial invasion by the leukemic cells after each inoculation.

The specific difference between lines U and S, indicated by the passive transfer of resistance to line U, is paralleled in the comparison of lines U and I. The passive transfer of line-I resistance has been effective against line I in 353 mice in 34 experiments, but against line U 184 out of 190 mice were not protected. Conversely, the passive transfer of line-U resistance protected 17 out of 20 against line U. In terms of the final result, line U may be said to have a capacity to immunize against itself comparable to that of line I, but the peculiarity of line I, and certain other lines, is the ease with which the first steps of active resistance can be accomplished. There is evidence of specific difference between these lines in their fully established resistance, but their different response to the StoLi treatment may depend upon some other mechanism, which determines the reaction of the host to very small doses. Any such separation of mechanisms implies the possibility of their independent variation.

The question concerning the importance of high virulence for the establishment of resistance appears to be answered in the negative. Line S has virulence comparable to line U, as judged by the intervals before death and by the characteristics of the autopsy picture; but, in spite of the larger number of tests and over a hundred more transfers, line S has shown no suggestion of susceptibility to StoLi treatment, nor, indeed, of the capacity to develop resistance passively transferable. An opportunity to test for such passive transfer was given by the exceptional survival of a mouse given a dose of S $4^{-3}$ times standard, which in the same experiment (CSH 8566) killed four litter mates of the same sex in six days. This mouse ( ♂ 78782) was reinoculated

with the same dose and then with five successive standard doses. That this mouse was fully resistant cannot be questioned, but when its liver was used to treat other hosts, none was protected. Four mice treated with 0.1 cc. and one with 0.2 cc. of the minced liver, together with five litter-mate controls, were inoculated with a dose of S $4^{-3}$ times standard; parallel groups of the same numbers were inoculated with S $4^{-4}$ times standard. With the larger test dose, the intervals before death averaged: treated, 7.40 days; controls, 7.35 days. With the smaller dose they averaged: treated, 8.05 days; controls, 8.10 days. No differences could be detected in spleen size or other gross lesions at death. The resistance of the mouse to line S differed in nature from that in the mice resistant to line U. That the resistance to line U could be passively transferred and that it was ineffective against line S, while resistance to line S was not transferred passively, indicate that, whatever characteristics these lines hold in common because of their origin in strain C58, they also have specific differences, and it is the existence of these specific differences that constitutes the problem.

### Resistance from Heated Leukemic Cells

A variation in the technique of inducing resistance employs heated leukemic cells (7). One standard dose of line I, heated to 46° C. for 10 minutes, enabled 90-100% of C58 hosts to resist the lethal dose, I $4^{-3}$ times standard, given after 20 days. The heat was applied in a circulating water bath, which during most of the experiments varied less than 0.03° C. on a Beckman thermometer. The leukemic-cell suspensions, in lots of 3 cc., were stirred for the first 2 minutes in the bath and after a standard exposure of 10 minutes removed to ice water. With all other conditions the same, the protective effect was eliminated by raising the temperature half a degree. One characteristic of all such experiments with heated cells was the all-or-none action upon individual mice; they either died at the same time as the controls or survived indefinitely.

A change in the reaction of line I to heat occurred in the course of the experiments, which employed transfers 829 to 1092. Table 2 shows a reduction in the proportion of survivors of the test dose, from 90% to 30%, beginning with transfer 914. Since line I was used both to induce and to test for resistance, the change in the outcome might have followed an altered reaction to the heat or an increase in the lethal capacity of the test dose. If the heat threshold for inactivation of the resistance had been lowered, a reduction in the heat treatment should restore resistance. Such a modification in the treatment was made and the resistance was restored.

The time in the 46° C. water bath was varied, rather than the temperature, in order to obtain more accurately controlled results. Samples of the same cell suspension were injected after 5-, 7-, and 10-minute exposures, and all the mice and their litter-mate controls were inoculated in 20 days with a lethal dose of the same suspension of untreated line-I cells. The reduction of the time in the water bath by five minutes, and even by as little as three minutes, resulted in every mouse's surviving the lethal test dose.

The evidence of this change in line I with respect to heat stands quite independently of any question of the nature of the process by which resistance was induced. But the assumption seems

TABLE 2. RESISTANCE INDUCED BY HEATED LEUKEMIC CELLS. TREATMENT: ONE STANDARD DOSE, LINE I, HEATED TO 46° C., FOLLOWED IN 20 DAYS BY LINE I, $4^{-3}$ OR $4^{-4}$ × STD.

| Line I transfers | Minutes in 46° C. water bath | Survived lethal doses ($4^{-3}$ or $4^{-4}$ × Std.) | |
|---|---|---|---|
| | | % | (total no.) |
| 829–879 | 10 | 90.5% | (127) |
| 914–934 | 10 | 29.6% | (54) |
| 1043–1092 | 10 | 26.4% | (87) |
| 1066–1092 | 5 or 7 | 100% | (74) |

reasonable that this process is intimately related to the active resistance establishable by very high dilutions of line I. Other lines with which parallel results from heat treatment can be obtained are those with which active resistance against themselves has been demonstrated. Thus line S, when heated, failed to give protection either against itself or, incidentally, against line I.

### SUMMARY

In the course of serial transplantation of leukemic cells in naturally susceptible hosts, persisting changes associated with these cells have been indicated by the following criteria: interval before death, autopsy picture, frequency distributions of cell and metaphase diameters, basophilia, rate of cell division, rate of infiltration, susceptibility of different inbred host strains and of segregating hybrids, reaction to a specific host gene, reaction to induced host resistance, and reaction to heat treatment. Undoubtedly all of these are not independent variables, but only in a few cases does a relationship between any of them appear to be directly dependent. In what way leukemic cells, or their structure, or their molecules or inclusions are related to the phenomena described is not discussed. It would seem premature at this stage to indulge in the speculation that is stimulated by the many parallels with phenomena, reported by other participants, in various types of microorganisms. This material is presented to indicate a field whose experimental analysis may find technical and theoretical guidance in the contributions of this Symposium.

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## DISCUSSION

Kidd: Among the phenomena that Dr. MacDowell and his associates have encountered, there are several that I would like to discuss further.

*Alterations in the behavior of transplanted cancer cells.* Dr. MacDowell and his associates have described in considerable detail an increase in virulence and an increase in host range—to follow their terminology—in several lines of lymphoma cells transplanted in C58 hosts. As we have just heard, all of the lines went through an initial period of stabilization, in which the survival time of the host animals became shorter and shorter in successive transplantations until a rather uniform level was reached, usually within the first dozen tumor generations. Following this the survival time of implanted hosts in the successive groups tended to remain fairly constant, though it very gradually diminished in some lines observed over long periods of time; occasionally, however, the lines underwent further and rather abrupt changes, killing their C58 hosts much more quickly than before, and manifesting the ability to grow in host animals of other inbred strains (StoLi). Practically every worker who has succeeded in establishing a transplantable neoplasm has noted that the recently transplanted cancer cells grew better and better in succeeding implantations—if indeed they grew at all, as often they did not in the days before inbred animals became available—while abrupt increases in the proliferative ability of transplanted cancers and in their ability to grow in animals of other lines have been studied in detail by Strong and Cloudman, among others.

It is plain that these variations in the behavior of transplanted cancer cells depend upon some alteration in the tumor cell-animal host relationship. Furthermore, there is every reason to believe that the less stable factor in this relationship is the newly transplanted tumor cell. For the most striking changes occur almost regularly and quite abruptly with each newly established line of tumor cells, as Dr. MacDowell's study of lymphomas in C58 mice has shown in illuminating detail, the animal hosts, already long inbred, manifesting no detectable changes meanwhile. What happens to make tumor cells grow better with each new transfer until a stable level is reached, usually within the first dozen transplantations? In order to comprehend the phenomenon it is necessary to recognize the fact that a striking difference exists in the cellular composition of autochthonous and transplanted cancers. Autochthonous cancers—that is to say, cancers composed of the animal's own cells (they are "spontaneous" only in the sense that we do not understand how they arise)—are usually made up of a spectrum of cells which differ notably from one another, though they are all of a single type; transplanted cancers by contrast are made up as a rule of standardized cells, each resembling the next as much as do the engines of modern automobiles of a single make. Is it not conceivable, in the light of what Dr. Sonneborn and I have said earlier this morning about distinctive cytoplasmic constituents of paramecia and cancer cells respectively, that the spectrum of cells making up autochthonous cancers should have differing amounts of *cancer kappa*—to coin a term embracing both types of cytoplasmic entities and implying that the cytoplasmic substance is responsible at least in part for the proliferation and autonomy of the cancer cells; upon transplantation only those cells would proliferate best which have the optimal amount of cancer kappa and the fewest iso-antigens to elicit resistance; within a few transfers the transplanted cancers would be composed of these cells alone, the others of the spectrum having been overgrown or outrun.

The idea could be extended to include the abrupt changes that take place occasionally in long-transplanted cancer cells, which result in increases in virulence, in growth energy, and in host range—phenomena previously studied by Strong and Cloudman and encountered also by Dr. MacDowell and his associates. For one would need only to imagine that the cancer kappa had been able to increase in amount rather abruptly, and had crowded out or suppressed normal constituents of the cell, the latter constituting iso-antigens whose presence had formerly elicited resistance when the cells were transplanted to hosts lacking them. It is possible that genic relationships might be involved in the hypothetical increment of cancer kappa, but the data of Dr. Sonneborn would seem to indicate that analogous happenings can take place in paramecia without involving genes except perhaps secondarily. As bearing further on all this, it is interesting to note that the changes in transplanted cancer cells observed by Strong, Cloudman, and MacDowell *et al.* were always in the direction of an increase in virulence and an increase in host range, never in the reverse direction, in which respect they resemble the phenomena of autonomy and anaplasia described this morning.

*Induced resistance against transplanted cancer in susceptible hosts.* Several years ago, Dr. MacDowell and his associates announced that they were able to immunize C58 mice against line-I leukemic cells (which had originated some years before in this long-inbred strain of animals and been transplanted constantly in it), by implanting into normal animals initially small but gradually increasing doses of the tumor cells; a certain proportion of the animals so treated failed to develop leukemia and later manifested immunity to massive doses of the leukemic cells. Later experiments showed that a preliminary injection of normal embryonic tissue cells from another inbred strain of mice (StoLi) rendered practically all C58 mice resistant to a small dose of line-I leukemic cells and that a large proportion of the latter could then be solidly immunized by means of increasing doses of the tumor cells; furthermore, the phenomenon could be repeated with two other lines of leukemic cells (designated as A and M-kid) which had likewise originated in C58 mice and been transplanted for many generations therein. The three lines of leukemic cells mentioned were found to be antigenically similar—that is to say, C58 hosts immunized against any one of the three strains were resistant to one or both of the other two, though it is noteworthy that the immunized mice manifested no resistance whatever to leukemic cells that had recently originated in C58 hosts.

Dr. MacDowell and his associates explained this phenomenon by assuming that the line-I (and lines A and M-kid) leukemic cells had varied during the course of the long-continued transplantations, acquiring presumably a new antigen or antigens capable of eliciting resistance in C58 hosts. May I suggest an alternate explanation: namely, that the leukemic cells had not acquired a new antigen during the long period of transplantation but that they had maintained an iso-antigen, which was present in C58 cells during the time when the line-I, line-A, and line-M-kid leukemic cells became malignant but which had since been lost from the cells of the inbred hosts—in other words, that the host animals had been bred away from the tumor cells?

It seems to me that tumor cells might very well continue to carry during long periods of asexual fission an unstable gene or genes (with associated antigen or antigens) that might more or less readily get lost from the gametes of host animals during the meioses of repeated sexual reproductions. The supposition that this happened in Dr. MacDowell's experiments has the merit of avoiding the assumption—awkward in the light of present-day genetic concepts—that homozygosity is long maintained in inbred mammals. And it avoids conflict with Haaland's dictum that an animal cannot be immunized against its own neoplastic cells—a principle which is notably reinforced by MacDowell's finding that C58 mice solidly immunized against line-I leukemic cells are quite as liable to develop "spontaneous" leukemia as are their untreated fellows, and one which is not notably weakened by the recent partial successes of Gross, of Goldfeder, and of Aptekman, Lewis, and King in attempts, with a variety of methods, to immunize animals of inbred strains against tumors that grow progressively in them. Furthermore, it avoids the improbable assumption—necessary if Dr. MacDowell's explanation is correct—that leukemic cells develop during long-continued transplantations an iso-antigen which is identical with that present in normal tissues of another strain of mice (StoLi).

Whether the antigen responsible for the resistance was formed as a result of variation in the leukemic cells during the course of transplantation, as Dr. MacDowell suggests, or whether it was maintained throughout the transplantations, as I believe—in either case it is plain that the antigen does not elicit an effective resistance in C58 mice under the ordinary conditions of transplantation where a relatively large dose of cells is implanted; for under these circumstances the cells "take" and grow progressively. The fact is not confounding, however: Gorer has pointed out that tumor cells frequently grow progressively in hosts having cells with an iso-antigenic make-up different from their own and lacking certain iso-antigens known to be present in the tumor cells.

Whatever its cause, the phenomenon of induced immunity to tumor cells long-transplanted in the inbred strain of origin, which Dr. MacDowell and his associates have uncovered, is hardly to be considered an example of a universal principle. For, al-

though other workers, as mentioned above, have reported partial successes in attempts to immunize animals against tumors that usually grow progressively in them, Dr. Helene Toolan and I were unsuccessful in recent attempts to accomplish this with a lymphosarcoma that had long been transplanted in C3H mice.

*Passive transfer of induced resistance to transplanted cancer cells.* For almost half a century the fact has been known that cancers transplanted to new hosts of the species of origin would in some individuals "take" and grow progressively while in others they would either fail to "take" or would grow briefly and then regress, the resistant hosts manifesting thereafter a complete immunity to further reimplantations with the same cancer. Needless to say, many attempts have been made to discern the mechanism whereby the tumor cells are overcome in the resistant hosts; yet to the present day, this mechanism remains largely obscure, except for two lines of evidence now to be mentioned.

The first has to do with the relation of humoral antibodies to the phenomena of resistance to transplanted cancer. Gorer and Lumsden showed several years ago that iso-antibodies may be found transiently in the blood of animals resistant to transplanted cancers; and the former worker has shown that "protective" antibodies can be stimulated, by injecting leukemic cells into mice naturally resistant to them, which are capable of suppressing the growth of the leukemic cells under appropriate experimental conditions.

The second bit of evidence has to do with the passive transfer of resistance to cancer. While the experiments of Dr. MacDowell and his associates on this theme have not yet been published in detail, it appears that they have been able to transfer resistance to line-I leukemic cells by injecting suspensions of living tissues (particularly liver) procured from immunized animals into normal C58 mice, the resistance thus passively transferred being transitory and comparatively weak and depending upon the transfer of living cells, serum from immunized animals being wholly ineffective. It is interesting that the observations of MacDowell *et al.* on the passive transfer of resistance to cancer have recently been paralleled almost exactly in experiments made by Merrill Chase, who has shown that hypersensitivity to simple chemical compounds and to tuberculin can be transferred passively from one animal to another by means of leukocytes procured from artificially inflamed peritoneal cavities of hypersensitized animals; in his experiments, too, living cells were necessary and serum was ineffective.

Much remains to be learned about the mechanism of resistance to cancer, and some convergence will be required to bring together the lines of evidence provided by the work of Gorer and Lumsden with humoral antibodies and by that of MacDowell and his associates on cell-induced resistance.

# BIOCHEMICAL STUDIES OF ENVIRONMENTAL FACTORS ESSENTIAL IN TRANSFORMATION OF PNEUMOCOCCAL TYPES

MACLYN McCARTY, HARRIETT E. TAYLOR,[1] AND O. T. AVERY

The phenomenon of transformation of pneumococcal types provides an outstanding example of the induction of specific and heritable modifications in microorganisms. Basically the phenomenon represents the transformation of a nonencapsulated (R) variant derived from one specific type of Pneumococcus into encapsulated (S) cells of heterologous specific type. By the technique employed at present, this is accomplished by growing the nonencapsulated cells in a special serum broth to which has been added the active fraction extracted from encapsulated pneumococci of a heterologous type. The production of a new polysaccharide capsule is induced in the R cells so that they acquire the type-specificity of the organisms from which the extract was obtained. The property of forming the new capsule is transmitted indefinitely to subsequent generations, and, in addition, the substance responsible for the induction of transformation is itself reduplicated in the transformed cells. It is thus apparent that one is dealing with hereditary bacterial modifications, which are predictable and subject to direct experimental control.

The chemical nature of the substance capable of bringing about this type of heritable change presents a problem of primary importance. Accumulated evidence based on the results of innumerable tests of the specificity and biological activity of various preparations, together with the data obtained by chemical, enzymatic, and serological analysis of the active material, has established beyond reasonable doubt that the active substance responsible for transformation is a specific nucleic acid of the desoxyribose type (2, 9, 10). These results suggest that nucleic acids in general may be endowed with biologically specific properties not hitherto demonstrable. Results confirming this view have been published recently in a preliminary report by Boivin and his co-workers on the role of desoxyribonucleic acid in inducing a transformation analogous to the pneumococcal phenomenon, using encapsulated strains of *E. coli* (3). These investigators state that evidence obtained by chemical and enzymatic techniques shows that desoxyribonucleic acid and not the protein of the nucleoprotein molecule is responsible for the specific effect. In so far as one can judge from this brief report, the results agree in principle with those obtained with Pneumococcus.

In the historical development of the problem of transformation it is of interest to recall that Griffith (6), who originally described the phenomenon *in vivo*, was unable to obtain positive results *in vitro*. The first successful demonstration of the reaction in the test tube was carried out by Dawson and Sia (5) in nutrient broth containing anti-R rabbit serum. From that time on, serum or serous fluid of one sort or another has always been used and has been shown to be an essential factor, since in its absence it is impossible to induce transformation. The function of serum is not merely one of enrichment of the culture medium, however, since the nutrient broth itself contains adequate amounts of accessory growth factors required for initiation and maintenance of growth. It is evident, then, that serum provides essential environmental factors for pneumococcal transformation *in vitro*, and clarification of the role played by the serum is of foremost importance in understanding the nature of the phenomenon. The following questions naturally arise: Why is the presence of serum or serous fluid in the medium essential? Why are some sera capable of supporting transformation while others utterly fail? What components function as essential factors, how do they act, and what is the biochemical nature of their action in respect to the cellular changes evoked by the specific pneumococcal nucleic acid?

Although anti-R rabbit serum was used in the initial studies, in recent years this has been largely supplanted by human chest or abdominal fluids which occur as the result of a variety of pathological processes. These serous fluids invariably contain more or less R antibody, but they show marked variations in their ability to support transformation, which are unrelated to the antibody titer. It has been observed, for example, that chest fluids accumulating as the result of mechanical factors, as in the case of cardiac decompensation, regularly have little or no activity, while fluids formed as the result of tuberculous or acute infectious processes are usually highly effective. This suggests that one of the essential serum components may be present only in very low concentration in normal sera, but may increase in the course of infectious disease. This empirical observation has served as a useful guide in selection of effective sera.

The results of studies on the role of serum in the transforming reaction indicate that at least three essential constituents are involved. These are: (1) the R antibody, which causes agglutination of unencapsulated R pneumococci; (2) a dialyzable con-

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stituent; and (3) a protein factor, in addition to the R antibody. Each of the three components is considered individually in the following discussion. The evidence for assuming that the serum factor depends on the collective action of the three components is summarized, together with a description of certain experiments designed to elucidate the function of each and the mechanism of their combined effect.

## THE ROLE OF R ANTIBODY

All sera that have proved effective in supporting transformation have contained R agglutinins, but despite this fact it was difficult to be certain that these antibodies were essential in the reaction. For example, there seems to be no relation between the titer of R antibody and the efficacy of the serum in the transforming system, and indeed some of the most potent sera have the lowest anti-R titers. The results of recent experiments, to be described below, provide some indication of the role of R antibody.

During growth in the serum broth used in the transformation system, the R cells agglutinate as they divide, so that each cell of the inoculum apparently gives rise to a colony, which becomes visible to the naked eye after several hours of growth (Fig. 1, a). Subsequently these colonial aggregates become larger and settle to the bottom of the tube, leaving a clear supernatant (Fig. 1, b).
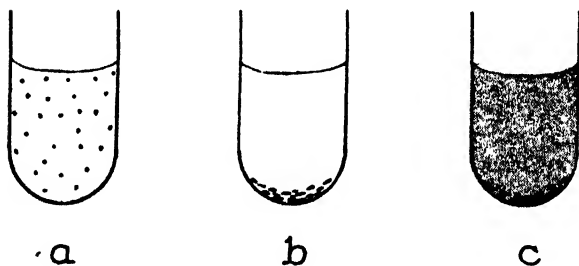


FIG. 1. Growth of pneumococci in serum broth. (a) Six-hour growth of R pneumococci. (b) Eighteen-hour growth of R pneumococci. (c) Transformation; 18-hour growth of R pneumococci in serum broth plus Type III transforming substance.

This latter fact is useful in the technique of the test, because newly formed S cells occurring as the result of transformation are not agglutinated by R antibody and grow diffusely throughout the tube (Fig. 1, c). Thus the appearance of the growth gives immediate presumptive evidence concerning the presence or absence of transformed pneumococci.

Colonial growth of R pneumococci somewhat analogous to that caused by anti-R results from the use of a semisolid medium (11). For example, in a viscous medium containing a low concentration of agar (0.2%) R pneumococci grow in loose aggre-

gates not unlike those formed by antibody agglutination. The growth differs, however, in that the colonies do not settle to the bottom of the tube (Fig. 2, a). S pneumococci give a fluffy, cotton-ball colony, which is readily distinguished from the R colony (Fig. 2, b), so that transformation in this type of semisolid medium can be recognized by the appearance of characteristic S outgrowths on the R colonies (Fig. 2, c). It has been found possible
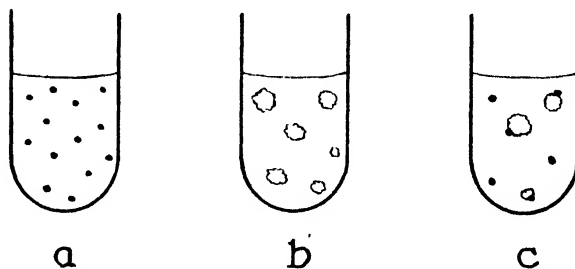


FIG. 2. Growth of pneumococci in semisolid medium. (a) Eighteen-hour growth of R pneumococci. (b) Eighteen-hour growth of S III pneumococci. (c) Transformation.

to bring about transformation in an agar semisolid medium containing normal rabbit serum but wholly lacking in R antibody. Neither normal rabbit serum nor the semisolid medium were by themselves capable of supporting transformation. This experiment suggests that the type of colonial growth produced by anti-R is an important factor and that when this type of growth is simulated by other means the anti-R can be dispensed with. It is important to note, however, that serum is required in the semisolid medium, although it is not essential that it contain R antibody.

Although it cannot be stated with certainty why colonial growth is required, it is possible that local reducing conditions arising in the aggregated cells are of primary importance. This thesis is supported by the results of experiments in which the medium is placed in a shallow layer not exceeding 1-2 mm. in depth. In the shallow layer, oxidizing conditions are promoted, and, even in the usual serum medium containing R antibody, manifest transformation does not occur. Attempts to reverse the effect of the shallow layer by the addition of reducing agents have not yielded consistent results, but on one occasion transformation was obtained in a group of flasks in which glutathione had been added to the usual serum medium. There is, then, some evidence that reducing conditions are essential in some phase of the transforming reaction.

On the basis of the evidence available at present it would appear that R antibody serves the purpose of causing an essential colonial aggregation of R pneumococci, which in turn results in local conditions, possibly reducing in character, that are required for transformation.

## DIALYZABLE SERUM CONSTITUENT

Early attempts at salt fractionation of serum factor by the classical methods of protein chemistry yielded totally inactive fractions. Some light has been thrown on these results by the discovery that a dialyzable constituent of the serum is essential. When an active serum is dialyzed against physiological saline, there is a progressive decrease in its efficacy in the transforming system, and if dialysis is sufficiently prolonged the serum becomes completely inactive. Under these conditions, however, the R antibody is unimpaired, and no denaturation of protein is apparent.

The period of dialysis required for complete inactivation varies for different sera from two days to three weeks. This suggests that the dialyzable substance may not be free in the serum but combined in loose linkage with a nondiffusible molecule. It is of interest, also, that sera are not inactivated by dialysis against distilled water, indicating that the salt ions may have some effect in displacing the dialyzable component from its linkage to the nondiffusible substance.

The problem of the nature of the dialyzable constituent has been approached by a study of the reactivation of serum rendered inactive by dialysis. Two distinct types of reactivation, which appear to be of different mechanism, have been devised. In the first place, if inorganic phosphate in concentrations of 0.005 M or above is added to dialyzed serum and the mixture incubated 1-2 hours, the phate mixture after treatment, as indicated in Table 1, and the serum medium was then tubed in 2.0-cc. amounts for the test of ability to support transformation. Type III transforming substance was added and the tubes inoculated with a diluted culture of a susceptible R strain derived from Pneumococcus Type II. The tubes were incubated and the occurrence of transformation determined by the methods described in previous communications from this laboratory.

The data given in Table 1 demonstrate that reactivation of dialyzed serum can be achieved with inorganic phosphate if sufficient time is allowed for interaction. A relatively slow reaction occurs, which may conceivably be enzymatic in character. In contrast to this procedure, it is possible to bring about immediate reactivation of dialyzed serum by the addition of such materials as unheated Neopeptone or tryptic digest of casein. Further investigation of substances causing this type of reactivation disclosed that inorganic pyrophosphate has a similar effect and is not influenced by the presence of nutrient broth. The effects of pyrophosphate and Neopeptone, as compared with the effect of phosphate, are illustrated in Table 2.

Sodium pyrophosphate was added to dialyzed serum in a final concentration of M/150, Neopeptone sterilized by filtration in a final concentration of 0.2%, and inorganic phosphate as in the previous experiment. Nine volumes of nutrient broth were added immediately and the test for ability to

TABLE 1. REACTIVATION OF DIALYZED SERUM BY INCUBATION WITH PHOSPHATE

| Source of Serum Factor | Additions | Period of Incubation before Addition of Nutrient Broth | Transforming Test with Type III Transforming Substance and Strain R36A Quadruplicate Tubes | | | |
|---|---|---|---|---|---|---|
| Dialyzed serum | None | 2 hrs. at 37° C. | R only* | R only | R only | R only |
| Dialyzed serum | Phosphate | 2 hrs. at 37° C. | S III* | S III | S III | S III |
| Dialyzed serum | Phosphate | None | R only | R only | R only | R only |
| Whole undialyzed serum | None | None | S III | S III | S III | S III |

* S III indicates the occurrence of transformation as evidenced by the recovery of encapsulated cells of Pneumococcus Type III, while the term "R only" means that transformation did not take place, and only unencapsulated R variants were recovered.

serum regains the ability to support transformation when added to broth in the usual concentration. The period of incubation of the serum with phosphate is essential. The interaction between phosphate and the serum appears to be prevented by the presence of nutrient broth, for if the latter is added at the same time as the phosphate, or after a short period of incubation, no reactivation is achieved. A protocol of an experiment demonstrating the reactivation of dialyzed serum by incubation with phosphate is given in Table 1.

The phosphate was added to dialyzed serum at a final concentration of M/150 in the form of $Na_2HPO_4$-$KH_2PO_4$ buffer, pH 7.8. Nine volumes of broth were added to the serum or serum-phos-support transformation carried out as before. The fact that pyrophosphate and Neopeptone restore the activity of dialyzed serum under conditions that are ineffective when simple inorganic phosphate is used is apparent from the data presented in Table 2. Transformation is as prompt in the reactivated serum medium as in the system containing undialyzed serum.

It is not known whether the effect of Neopeptone is dependent on the presence of inorganic pyrophosphate or whether other substances, such as certain organic phosphates, are perhaps also capable of reactivating dialyzed serum. A few organic phosphates, including adenosine triphosphate, pyridoxal phosphate, glycerophosphate, and fructose

diphosphate have been shown to be without effect.

The difference in the action of phosphate and pyrophosphate is emphasized in the case of globulin fractions of active sera. These fractions have for the most part been prepared by half saturation with ammonium sulfate followed by dialysis of the redissolved precipitate to remove excess sulfate ion. Globulin fractions so obtained are totally inactive in the transforming system, and even prolonged incubation with inorganic phosphate does not serve to reactivate them. On the other hand, immediate reactivation results from the addition of either in-

that some organ of the animal body contains the enzyme in much higher concentration than does serum and would serve as a more favorable source for possible purification and identification of the enzyme. To test this assumption, a preliminary survey was made of several rabbit organs by preparing simple saline extracts and testing them for the presence of the third component. The procedure used consisted of adding the extract to broth containing a small amount of concentrated rabbit R antibody plus unheated Neopeptone as a source of the dialyzable constituent. The broth containing these

TABLE 2. Immediate Reactivation of Dialyzed Serum by Pyrophosphate and Neopeptone

| Source of Serum Factor | Additions | Treatment | Transforming Test with Type III Transforming Substance and Strain R36A Quadruplicate Tubes | | | |
|---|---|---|---|---|---|---|
| Dialyzed serum | None | | R only* | R only | R only | R only |
| Dialyzed serum | Phosphate | Nutrient broth | R only | R only | R only | R only |
| Dialyzed serum | Pyrophosphate | added immediately | S III* | S III | S III | S III |
| Dialyzed serum | Neopeptone | to all tubes. | S III | S III | S III | S III |
| Whole undialyzed serum | None | | S III | S III | S III | S III |

* Symbols same as in Table 1.

organic pyrophosphate or Neopeptone. It appears that salt fractionation removes an unknown substance that is essential in the reaction by which phosphate effects reactivation but plays no part in the pyrophosphate reaction.

It seems inescapable that phosphate has an important function in the action of serum factor. Whether this effect is direct or indirect has not yet been conclusively determined. An example of an indirect action of phosphate in an enzymatic reaction has recently been provided by the work of Colowick and Price (4), who showed that phosphate brings about the reactivation of dialyzed preparations of muscle hexokinase. In this case, the phosphate is involved in a separate enzyme reaction which results in the release of guanine, an essential coenzyme for hexokinase activity.

### The Third Component of Serum

The activity of serum in supporting transformation does not depend solely on R antibody and the dialyzable component. This is demonstrated by the fact that purified R antibody is not effective in the transforming system even when fortified by the addition of pyrophosphate or Neopeptone to provide the dialyzable component. The third component of serum is a nondialyzable substance, which has not yet been sufficiently characterized to establish its chemical identity. However, the results of fractionation procedures indicate that it is protein in nature.

For the purpose of orienting further research, the possibility has been considered that the third factor may be an enzyme. If this is indeed the case, it seems highly probable on general grounds

added components was tested for its ability to support transformation. Positive results indicate that the organ extract has supplied the missing constituent, since as pointed out above, R antibody and dialyzable factor alone are unable to support transformation. Rabbit spleen proved to be a good source of the third component. To provide larger organs as source material, extracts of calf spleen and calf thymus were then tried, and it was found that thymus extracts were more active than those of any of the other organs tested. The apparent superiority of thymus extracts may be due in part to the almost complete absence of desoxyribonuclease, which inactivates the specific transforming substance and thus interferes with the test. Although these preliminary experiments have demonstrated that the third component is present in certain mammalian organs, little progress has been made in the isolation of this substance. Thymus extracts have proved to be exceedingly difficult to handle in fractionation attempts, because of the presence of mucoid material. Nevertheless, it is likely that a more favorable source will be found so that purified preparation of this component of the transformation system can be obtained and analyzed.

### The Occurrence of an R Variant Not Requiring Dialyzable Component for Transformation

It has become increasingly apparent in recent years that bacterial populations, in common with populations of other living organisms, constantly undergo discontinuous variations, which must be presumed to be of the nature of genetic mutations. Inevitably, spontaneous variation of this sort be-

comes involved in a problem like that of pneumococcal transformation, in which relatively large bacterial populations are employed. The importance of mutation, or, as it is commonly called by microbiologists, dissociation, has been previously pointed out in connection with one aspect of this problem (2). The strain of R pneumococcus (R36A) derived from Type II which is used in the majority of transformation experiments in this laboratory gives rise to numerous variants recognizable in a general way by slight alterations in colony topography. Certain of these variants acquire significance by virtue of the fact that they are totally insusceptible to the effects of the transforming substance. In all, four distinct variants of the parent R strain have been isolated which are not responsive to the influence of the transformation substance. Despite the fact that the R strain is cultivated continuously under more or less uniform conditions, subject to the limitations imposed by the complexity of the medium required for growth, the incidence of this type of dissociation is variable. This is presumably due to undefined differences in environmental conditions, which result in changes in the selective properties for a given mutant.

Another variant of strain R36A has been encountered, which is of special interest in connection with the dialyzable component of serum factor. This R variant, designated 6e, was originally isolated from a culture in a medium containing dialyzed serum, which appeared to be deficient for growth, since only a few of the R cells of the inoculum multiplied to form visible colonial aggregates. Strain 6e has been found to be not only susceptible in the transforming system, but completely independent of the presence of the dialyzable component of serum factor. Thus, it undergoes transformation readily in systems that contain dialyzed serum or globulin fractions of serum, without the addition of dialyzable component in the form of pyrophosphate or Neopeptone. Strain 6e therefore behaves in all respects as though it supplies its own dialyzable component or effects restoration of the serum by some analogous reaction. The protein factor is required, however, since transformation cannot be effected when only the R antibody is present in the system.

Strain 6e is of potential value in further study of the serum factors. Attempts have been made to determine in what way it differs from the parent strain with respect to synthesis or release of a substance replacing the dialyzable serum component. It has not been possible to show that larger amounts of such a substance accumulate either in the cells or in the supernatant medium of cultures of the variant.

### EXPERIMENTS ON THE MECHANISM OF ACTION OF SERUM FACTOR

A series of experiments, which were designed to provide a more intimate knowledge of the interaction between the specific transforming substance (pneumococcal desoxyribonucleic acid) and the susceptible pneumococcal cells, proved to have an important bearing on the problem of the role of serum factor. The customary procedure in demonstrating the phenomenon of transformation is to add the specific desoxyribonucleic acid to the serum medium and to inoculate with a susceptible strain of R pneumococcus. Transformation becomes apparent after 16 to 20 hours' incubation, but little is known of the course of events during this period of incubation. The purified enzyme, desoxyribonuclease, which specifically inactivates the transforming substance (8, 9), has been used as a tool in an attempt to study certain phases of this problem.

TABLE 3. THE USE OF DESOXYRIBONUCLEASE IN DETERMINING THE TIME REQUIRED FOR THE UPTAKE OF TRANSFORMING SUBSTANCE BY SUSCEPTIBLE CELLS

| Time of Addition of Enzyme after Inoculation | Transformation Test | | | | |
|---|---|---|---|---|---|
| | Active enzyme; triplicate tubes | | | No enzyme; duplicate tubes | |
| 1 hour | R only* | R only | R only | S III* | S III |
| 2 hours | R only | R only | R only | S III | S III |
| 3 hours | R only | R only | R only | S III | S III |
| 4 hours | S III | S III | S III | S III | S III |
| 5 hours | S III | S III | S III | S III | S III |
| 6 hours | S III | S III | S III | S III | S III |

\* Symbols same as in Table 1.

By adding desoxyribonuclease to the transforming system at various intervals after inoculation in a concentration known to cause almost immediate inactivation of the transforming substance, it is possible to determine the length of time required for the transforming substance to be taken up or "fixed" by the susceptible cells. An experiment of this type is illustrated in Table 3.

An amount of Type III transforming substance representing 1000 minimal effective doses was added to each of 30 tubes containing 2.0 cc. of serum medium. The tubes were all inoculated with 0.05 cc. of a suitable dilution (containing approximately 1000 cells) of the susceptible R strain, R36A, and then incubated at 37° C. At hourly intervals groups of five tubes were treated in the following way. To three of the tubes were added 4.0 μg. of purified desoxyribonuclease in 0.2 cc. of nutrient broth containing 0.03 M $MgSO_4$; and to the remaining two tubes was added 0.2 cc. of the $MgSO_4$ broth without enzyme, as control on the effect of the agitation required to mix the enzyme thoroughly with the culture. The cultures were incubated overnight and the presence of transformation determined by

the usual procedure. The results are recorded in Table 3.

It will be seen that the addition of desoxyribonuclease at any time up to four hours after inoculation interferes with the reaction so that transformation does not occur, and it is therefore likely that throughout this period the transforming substance is readily accessible to the action of the enzyme. After four hours, on the other hand, the addition of desoxyribonuclease has no observable effect on the course of the reaction. This type of experiment was repeated with essentially the same results on several occasions and with different sera. Consequently, it appears that growth of the R cells in serum medium for 3 to 5 hours is required before the specific desoxyribonucleic acid is taken up by the cells and thus protected from enzymatic destruction. Further experiments have demonstrated that this does not depend solely on the increase in population (from the original inoculum of 1000 cells to an approximate 1,000,000 cells at 4 hours) and the consequent appearance of susceptible variants.

Confirmation of the importance of the four-hour period of growth is provided by experiments in which the R cells are grown in serum medium in the absence of the specific transforming substance. After 4 to 5 hours' growth under these conditions the cells are so "sensitized" that, when they are transferred to a medium containing the transforming substance, the latter is taken up in as short a time as 15 minutes. That is, desoxyribonuclease has no effect on the outcome of the transforming test when added 15 minutes after the previously "sensitized" cells are brought into the presence of the specific desoxyribonucleic acid. If the transfer is made after shorter periods of growth in the serum medium—e.g., 2 to 3 hours—the "sensitization" has apparently not taken place and rapid fixation of the transforming substance cannot be demonstrated. Furthermore, if growth in the serum medium is prolonged 7-8 hours or more the "sensitization" is lost, indicating that the alteration in the cells is not permanent but is affected by further environmental changes that occur on continued growth. These experiments demonstrate that the events which occur in the first four hours of growth of R cells in the transforming system are independent of the presence of the specific transforming substance. It must be concluded that growth under these conditions alters the cell in some· way, or provides suitable environmental conditions, so that interaction between the cell and transforming substance can take place.

The relation of these experiments to the role of serum factor becomes apparent from the fact that a complete and active serum containing all the essential components muse be present in order to achieve "sensitization" of the cells during the four-hour period. Growth in the presence of purified R antibody, in serum inactivated by dialysis, or in globulin fractions of active sera, fails in each case to "sensitize" the cells. Thus the hypothesis is suggested that the major part played by serum in the transforming system is concerned with a modification of the R cell so that the specific transforming substance can be taken up.

## DISCUSSION

It seems well established that the environmental conditions required for transformation of pneumococcal types *in vitro* depend on the combined action of several distinct factors. The present studies fall far short of the ultimate goal of determining the chemical nature and mode of action of the various factors involved. However, they provide a basis for and give direction to further research and make possible the formulation of a tentative hypothesis concerning the role played by serum in the transforming reaction.

The experiments in which desoxyribonuclease was used to inactivate the transforming substance free in the reaction system have demonstrated that the specific desoxyribonucleic acid does not participate in the preliminary phase of "sensitization" which takes place during the first four hours. On the other hand, the three known components provided by serum or serous fluid are all required during this four-hour period. At present, the most reasonable interpretation of the available data is that the action of the serum factors during this early phase results in alterations at the surface of the R cells so that they are capable of taking up or adsorbing the specific transforming substance. The alternative interpretation that the serum provides a strongly selective environment for growth of R mutants susceptible to transformation has been discarded on the basis of several important considerations. First, "sensitization" takes place in a relatively short time and is not dependent to any marked degree on the size of the original inoculum or on the total population. During the last 30 minutes of the four-hour period the number of "sensitized" cells increases from zero to at least 5000, representing approximately 0.5% of the total population. The final and most important evidence against the assumption that selection of a mutant is involved depends on the fact that the state of "sensitization" is temporary and readily lost. Cells that have been "sensitized" by growth for 4 hours in serum medium can be deprived of their acquired "sensitization" by repeated washing with nutrient broth or simply by allowing them to grow an additional 2 to 4 hours in the serum medium.

If it is assumed, then, that the action of the environmental factors is exerted at the surface of the R cells, the problem resolves itself into one of determining the nature of the alteration of the cell surface and the way in which the serum components bring it about. Perhaps the most attractive hypothesis is that the reaction is enzymatic in char-

acter and is dependent upon the action of an un-identified enzyme, represented by the third component of the serum, on certain specific groupings at the cell surface. It is well established that profound but temporary alterations in the peripheral mosaic of living bacterial cells can be brought about by enzymatic action. For example, the bacterial enzyme that hydrolyzes the capsular polysaccharide of Pneumococcus Type III is capable of removing the capsule from viable Type III organisms (*1*). The functions of capsule formation and polysaccharide synthesis are not impaired, however, and in the absence of the active enzyme, descendants of cells so treated again have all the characteristics of normal Type III cells. A similar phenomenon has been described in the case of hemolytic streptococci, from which one of the surface antigens, the M protein, can be removed by the use of proteolytic enzymes without affecting the viability of the bacteria (*7*). Therefore, in the case of the transforming reaction, it is not unreasonable to suppose that less extensive reversible alterations at specific sites of the surface of pneumococcal cells can result from enzymatic action, and that these alterations make possible the adsorption or penetration of the specific desoxyribonucleic acid.

In terms of the enzyme hypothesis, the occurrence of a dialyzable constituent is referable to a dissociable cofactor. The reactivation of dialyzed serum by incubation with phosphate has its counterpart in the work of Colowick and Price (*4*) in which the hexokinase activity of dialyzed muscle extracts is restored by incubation with phosphate. In this latter case, the release of a cofactor, guanine, by a secondary enzymatic reaction involving ribonucleic acid is responsible for reactivation. However, in the restoration of the transforming activity of dialyzed serum the immediate effect of pyrophosphate suggests that a different type of reaction is involved than in the case of hexokinase.

The function of the essential colonial aggregation of the R cells resulting from the action of R antibody is more difficult to interpret from the point of view of the enzyme hypothesis. However, bacterial aggregation may have an important relation to the period of growth required for "sensitization" of the cells, since at four hours the individual aggregates are just beginning to reach a size (about 1000 cells) which could provide special local conditions. It is conceivable that these local environmental changes, reducing or otherwise, provide essential conditions for the action of the enzyme. Thus, it is not necessary to make the unlikely assumption that the preliminary four-hour period is required because of the slow action of the hypothetical enzyme.

It scarcely need be reiterated that these considerations are tentative in character and subject to modification in the light of future results. How-ever, the various assumptions discussed above provide the basis for a useful working hypothesis, the validity of which can be further tested experimentally. The results of the present studies leave little doubt that the contribution of serum to the transformation system is complex and dependent on at least three components: the R antibody, an additional protein component, and a dialyzable factor which may be combined with the protein component as it occurs naturally. Furthermore, evidence is presented which strongly suggests that the role of serum is concerned with alteration of the surface of the R cells so that the specific desoxyribonucleic acid is taken up or adsorbed.

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# THE STATE OF VIRUSES IN THE INFECTED CELL

## N. W. PIRIE

When it has been established that a characteristic set of symptoms in an organism is due to an infective state, it is unfortunately sometimes assumed that the cause is a virus simply because no other causative agent can be demonstrated. The assumption becomes plausible if symptoms regularly associated with accepted virus infections—*e.g.*, inclusion bodies—are found, but it can be looked on as established only if infection can be produced by extracts that are free from intact infected cells. The ideal virus disease is one that can be transmitted by an extract that is free from cells or gross fragments of cells. This restriction excludes some accepted animal viruses and those plant viruses that can be transmitted only by grafting or by insect vectors; but, for the present purpose, a definition that is too narrow is preferable to one that is too broad. The transmission of infection in this ideal case can occur only if the infection leads to the production, not only of further virus, but also of virus unattached to the cell structure. It is clear that unless part of the virus can be in this state a lesion either would not spread or would spread extremely slowly. But the amount of what may be loosely called "free" virus, present at any one time, may be extremely small—so small as to be unrecognizable in extracts. A cumulative process would then be necessary for its recognition, and the opportunity for such a cumulative process may occur after grafting.

Cell-free extracts are also essential before any direct study of the intrinsic properties of a virus can be undertaken; for, although microscopical observations have thrown much light on the mechanism of virus infection, the interpretation of observations on the infected host depends in part on a real or assumed knowledge of the properties of the isolated virus. The properties of infective preparations made from these extracts do not, however, necessarily give information about the properties of the virus as it exists in the cell. There is no reason to doubt that material, resembling in a general way the principal component of some of the virus preparations that have been made, is infective; it is less certain that any or all of the free virus in the cell has the same properties as this material. There are three main reasons for this uncertainty:

(a) The preparation may be contaminated with constituents of the normal host having properties superficially so similar to those of the virus that the rather crude methods of fractionation at present available fail to separate them. (b) Each individual virus particle may be associated as a complex with constituents of the host that are not essential for its activity. (c) The process of fractionation may alter some of the properties of the virus without robbing it of all its infectivity.

A consideration of the first reason is of little interest, for the difficulty is purely technical and further work will presumably remove it; but the other two involve many points of general interest and importance.

### COMPLEXES WITH COMPONENTS OF NORMAL TISSUE

Chester (*11*) showed, by the anaphylactic response reaction, that the early preparations that contained tobacco mosaic virus also contained material that reacted like normal leaf protein. This material could be removed by digestion with trypsin (*3*), but the treatment caused other changes in the particle. Few virus preparations have been examined critically for this type of contamination; but freedom from normal leaf protein has never been demonstrated with a preparation that remains in a state at all comparable to the probable state of most of the "free" virus of the leaf. The anaphylactic test is not suitable for making a precise estimate of the amount of normal leaf material present, and its sensitivity is such that a strong reaction may be given when there is only 5% of contaminating material present. Such a quantity might well exist as independent particles having properties unlike those of the virus; for the techniques usually adopted for demonstrating homogeneity are not well adapted for the recognition of low levels of contamination (*26*). The normal leaf material may, however, be attached to the virus particle; and recent studies on virus preparations, in different states of aggregation, made by methods designed to cause as little modification as possible (*6*), make this appear probable. Tobacco mosaic virus, when prepared by ultracentrifugation or by precipitation methods, is only partly aggregated. A product that Bawden and Pirie (*6*) refer to as fully aggregated can be made by incubation with trypsin; the chemical and physical properties of this material are so characteristic that the ultimate recognition of aggregated virus is the best criterion of the earlier presence of virus in one of its less aggregated forms. The least aggregated particles are associated with their own weight, or more, of material that cannot be converted by digestion with trypsin into nucleoprotein having the properties of aggregated tobacco mosaic virus. In more aggregated fractions there is less of this material, and its removal is accompanied by further aggregation. The evidence here is by no

means complete, and it has not been demonstrated that the extra material is related, serologically or otherwise, to constituents of the normal leaf. It is clear, however, that the use of gentle procedures in the isolation of tobacco mosaic virus leads to a product that is chemically more complex than that made by more rigorous methods, and it is probable that the extra material is combined with the virus and not simply mixed with it.

The state of affairs with the influenza viruses is in many ways similar. Knight (21) finds that virus derived from the allantoic fluid of the embryo chick will precipitate with antiserum made against normal allantoic fluid, but that virus prepared from mouse lungs will not. Conversely a virus preparation from mouse lungs precipitates with anti-lung serum but not with anti-allantoic fluid serum. The evidence suggests that normal protein is actually combined with the virus, for this can be precipitated by antisera from animals injected only with normal tissue extracts. Furthermore, there is little difference in the intensities of the action when crude, obviously contaminated preparations and rigorously purified, apparently homogeneous preparations are used. It seems, therefore, that the virus associates with some of the components of whatever environment it has grown in, but that these—since different environments contribute different substances—are not specifically essential for the virus activity. There is no published information on the properties of preparations that have been shown to be free from this replaceable material.

Interesting parallels to these phenomena have been found with the pneumonia virus of mice. The virus can be recognized not only by the ability to cause characteristic infection but also by the ability, which it shares with some other viruses, of agglutinating red corpuscles. Mills and Dochez (23) found that the virus present in the fluid exuding from the cut surfaces of infected lungs carried both these activities, but that if the lungs were ground red-cell agglutination could only be demonstrated after the extracts had been heated. Careful investigation by Curnen and Horsfall (13) gives a satisfactory explanation of this difference. There is a substance in lungs which readily combines with the "free" virus, causing at the same time aggregation and the loss of the ability to agglutinate corpuscles. This substance does not, however, come into contact with the virus *in vivo* but only during the process of grinding; and the combination can be avoided by suitable extraction procedures. The complex can also be dissociated by heating or exposure to alkalis, but these treatments lead to loss of infectivity although they restore the ability to agglutinate red cells. There is no evidence on the nature of this complex-forming agent, but extracts containing it can be made from normal lungs.

These three viruses show comparable potentialities; but the differences in their behavior, in the environments in which they actually occur, also deserve emphasis. At least part of the pneumonia virus exists in the "free" form with a diameter of about 30 m$\mu$. This aggregates and forms a complex of diameter 100 to 150 m$\mu$ with a lung constituent. Tobacco mosaic virus, on the other hand, is protected from aggregation by a normal leaf constituent; and Bawden and Pirie (6) suggest that it can exist in an unaggregated form only when protected in some such manner as this. With influenza there is no clear evidence; preparations consisting of particles of the accepted size, about 100 m$\mu$, have never been shown to be free from the normal tissue component, and the small particles present in some virus preparations are not infective though they carry some of the activities of the virus. The opposite roles played by the normal tissue component in the cases of pneumonia virus and tobacco mosaic virus make it hard to decide whether or not, in the latter case as well as in the former, the complex is an artifact produced during the process of extraction. Complexes with normal leaf components can be made by intense grinding (4), but there is no evidence that they form, with consequent disaggregation, when infected leaves are treated gently or when virus and normal leaf extracts are simply mixed. Aggregations of differing degrees of firmness have, however, been demonstrated (6), and in all the preparations that have been tested aggregation was already extensive and stable. The behavior of a strawberry virus is in some ways analogous to that of the pneumonia virus, for here leaf extracts contain so much tannin that protein added to the extract is precipitated (1). The apparent absence of virus from extracts of infected leaves is not surprising therefore. In this case, as in that of virus pneumonia, the virus and the substance that combines with it may occur in different cells, so that they do not meet until the tissue is macerated. They may, on the other hand, be in the same cell but held apart by its internal architecture. There is no evidence that would seem to make one interpretation more probable than the other.

The important point established by these observations is that viruses react with other tissue components, and this must be kept in mind in assessing the validity of all existing evidence on the properties of viruses. Even in the original unfractionated extract the virus may be in a different state from that in which it exists in the cell. All those components of a preparation that are not invariably present when the preparation is infective may be looked on as contaminants, even though the presence of some form of replaceable material is essential to maintain the stability or primary state of dispersion of the virus. These contaminants are frequently present in preparations at early stages in the fractionation; the possibility of their removal depends mainly on the intrinsic stability of the virus. With stable viruses, such as

tobacco mosaic virus, a wider range of treatments can be applied than is possible with the unstable viruses.

### ALTERATIONS DURING ISOLATION

A rigid distinction cannot be drawn between the changes associated with the formation and destruction of complexes with normal tissue components and the processes of aggregation or disaggregation that some viruses undergo during purification. The evidence for aggregation generally rests on the appearance of particles of various sizes in the final product or on a change in the average particle size during the course of a preparation. The former effect could with equal probability be due to a variation in the size of the particles produced at the original site of virus synthesis, and it is probable that this is the cause of the observed variability in some of the larger viruses. This variability has rightly been emphasized by Beard and his colleagues (*9*), and they conclude that it is therefore necessary to look on viruses as small organisms with a cycle of growth and reproduction. This conclusion seems to ignore the meagerness of our information about the homogeneity of other large natural aggregates. There is no evidence that the particles of starch, glycogen, or haemocyanin are any more uniform than those of viruses of comparable size have been shown to be, yet no one proposes an organismal mode of reproduction for them. Nothing that is known about the synthesis of large molecules in normal tissue compels us to assume that the products of the synthesis are identical. The basic problem is not so much whether viruses can most usefully be called molecules or organisms but whether particles in the range generally referred to as "macromolecular" can usefully be called molecules. The problem is inescapable when particles 20 to 100 m$\mu$ wide are under consideration but it is already present when the dimension is only 2 to 10 m$\mu$ (*26*).

The antithesis between molecules, in the classical sense of the organic chemist, and organisms, as understood by the bacteriologist, is falsely drawn. There is an extensive zone in which "particle" is a safer word and where the dimensions that can be associated with the same activity are elastic. It is generally agreed that organisms can manifest their infective and other properties although covering a range of sizes. The assumption that this ·is also true of viruses is implicit in the use of their variability in size as evidence that they should be called organisms. It is not obvious why a similar elasticity should be forbidden a particle that is produced by the infected tissue or with its cooperation or by whatever other mechanism the smaller viruses are produced.

One disadvantage of the common practice of referring to the smaller viruses as molecules and of using such phrases as molecular weight in connection with them is that it leads to an attitude of mind in which it is assumed that all the particles in a preparation are identical. This is a foolish assumption, for it is unnecessary and, with particles of the size with which we are concerned, there is no way of demonstrating whether it is true or not. In a small molecule the addition or removal of a single atom or of a -CH$_2$- group makes a noticeable difference, because the change involves a significant percentage of the whole molecule. There is no reason to think that such a change, if affecting the less polar parts of the structure, would be observable in even a small protein. The assumption that the size and composition of a protein, characterized by the possession of a specific activity, is necessarily unique is an extrapolation from the inevitable state of affairs with the smaller and better-understood molecules. With particles as large as viruses are known to be, this technical limitation is even more important than with normal proteins. No measurement that has so far been made on a virus would detect variations of as much as 100,000 molecular-weight units among the particles composing the preparation that is being studied. Under certain circumstances the unobservable variability could be very much larger.

The most obvious change caused by the process of isolation, and the one that has provoked most discussion, is the aggregation that proceeds in tobacco mosaic virus preparations during purification or even on standing. It is very probable that part of the virus in the infected cell is already in an aggregated, or partly aggregated, state. The proportion that is aggregated, however, is increased by most of the processes used in purification (*2*). The virus, both outside and inside the cell, has therefore a range of properties, and it becomes meaningless to speak categorically of the properties of this virus. In the expressed sap from leaves two groups of substances affecting aggregation have been recognized. In the first place there are materials of low molecular weight that diffuse readily out of cells in which osmotic control has been damaged by freezing but in which the cell wall has not actually been broken. These cause aggregation. The indiffusible components of the normal leaf that are associated with the virus in crude preparations have already been discussed. They impede aggregation. The average length of the particles in a preparation depends on the past history and present environment of the preparation. It is a compromise between the tendency towards aggregation and the forces, such as Brownian movement, that tend to disperse the aggregates. The point at which a balance is struck depends on the extent to which protective substances are allowed to remain with the virus. The available evidence, though not conclusive, tends to confirm Bawden and Pirie's (*2*) early view that the virus that finally appears in solution in the expressed sap exists in the infected cell for the most

part in a spherical or at least not greatly elongated form. This evidence is derived not only from a study of the state of the virus in extracts made by different methods and of the changes that it undergoes in them but also from a crystallographic interpretation of the structure of the crystalline inclusion bodies that are a characteristic feature of infection with tobacco mosaic virus (*10*).

Similar aggregation has been demonstrated or suggested with several other viruses; among the plant viruses, potato "X" and "Y" and hyoscamus virus 3 may be mentioned. Gard (*17*) finds that poliomyelitis virus, and some viruses closely related to it, aggregate during purification, but the phenomenon has not yet been studied in detail. Little light is thrown on the state of the virus in infected cells, for it is not possible to study the virus until after it has been subjected to treatments that may well cause aggregation. Virus that is already free in the feces appears to behave in much the same way as virus extracted from infected brains.

Influenza viruses have for some years been looked on as spherical or nearly spherical particles, and most preparations undoubtedly consist largely of these particles. Rods, some formed apparently by the stringing together and some by the fusion of these particles, have now been recognized in preparations by Mosley and Wyckoff (*24*). These observations were made on virus after ultracentrifugal separation from the allantoic fluid of infected chicken embryos and drying to get a film suitable for electron micrography, so that the relationship between the elongated forms and the virus as it occurs in the infected cell is uncertain; but they clearly show that aggregation is one of the attributes of the particles in the preparation. Aggregation has also been described during the purification of some bacterial viruses, but definite evidence for this has not been published.

Aggregation is therefore a frequent concomitant of the purification of viruses, but it is not necessarily an invariable one. Tomato bushy stunt virus, for example, has been made from different plants and by different techniques, but no forms aggregated in solution have been recognized. It is possible that part of the virus in the infected cell has different properties from those normally ascribed to tomato bushy stunt (*24, 8*) but no phenomena at all comparable with those described for other viruses have been found. Most viruses have not been studied sufficiently closely for the absence of evidence of aggregation in them to be significant.

It has long been known that the infectivity of a virus preparation could be destroyed without there being any other observed change in its properties. Since mixtures of infective and noninfective material proved inseparable, it was obvious that, in the absence of other evidence, the greater part of the preparation might be noninfective material. During the course of purification there is generally a progressive fall in the apparent infectivity of a virus preparation, though in the early stages of the purification of a plant virus this may be masked by an increase in the apparent infectivity brought about by the removal of leaf constituents that reduce the infectivity. When an estimate of the weight of a virus particle can be made, infectivity measurements set an upper limit to the extent to which a preparation can be contaminated with noninfective particles. At least one infective particle is necessarily involved in the establishment of each discrete point of infection, whether this is an individual infected organism or a localized lesion. With several animal viruses, preparations have been made that will cause infection when only one to ten particles are used, but with plant viruses thousands of particles are needed even with the best preparations. Successive preparations may differ significantly in their infectivity although the same methods have been used in their preparation. This suggests that not all of the noninfective particles—whose presence in all virus preparations it is still reasonable to presume—are made during the course of isolation, but that some are already present in the infected plant. The progressive loss of infectivity remains, however, one of the more serious of the alterations that a virus suffers during isolation. There is no information about the probable nature of the change.

### ATTACHMENT OF VIRUSES TO THE CELL STRUCTURE

If much of the virus in an infected cell is not in the "free" state, a study that is limited to the part that is "free" may be extremely misleading. There is now abundant evidence that viruses are attached to the cell structure, and it is reasonable to consider this evidence under two headings: (a) virus that is still attached to the cell in which it was produced; (b) virus that has come from outside the cell and has attached itself as the first step in the establishment of infection.

The first state cannot be rigidly distinguished from the associations between viruses and normal tissue constituents that have already been discussed; the difference simply depends on the situation of the material to which the virus is attached. There is, however, a difference in the interpretations that can be put on the phenomenon. The association of a virus with tissue fragments may be due to its entanglement in parts of the morphological structure, and its separation from this may be due to more purely mechanical processes than those involved in its separation from the smaller tissue components present in extracts. The inclusion bodies, "X" bodies, and intracellular crystals that characterize many virus-infected cells are intermediate; for here the structure is large enough to be visible microscopically, but it is so fragile that the usual processes of extraction would disperse it

readily. In any event the total proportion of the virus in the cell that is present in these visible forms is small in those viruses that have been most thoroughly studied. There are exceptions, however; thus the polyhedral diseases of insects and some virus-induced warts lead to the accumulation in the cell of large masses of material that is thought to be at least in part virus.

Normal tissues contain a group of histologically recognizable bodies, as well as some smaller ones such as the microsomes of Claude (12), that can be prepared by processes of grinding, extraction, and centrifugation similar to those used in making a virus preparation. It has for long been clear that many virus preparations are contaminated by simple admixture of these cytoplasmic granules, for they fall in much the same size range as some viruses. The growing body of evidence that enzymes are associated with the granules has suggested that viruses also may be associated with them. So far there is no evidence for this, but Kidd (20) finds that microsomes from the Brown-Pearce tumor of rabbits contain a noninfective antigen that is characteristic of the tumor and induces the formation of antibodies that inhibit tumor growth. The occurrence in infected tissue of a noninfective material antigenically related to the virus is similar in many respects to the phenomena that have already been described with tobacco mosaic virus. As in that case, however, the relationship between the antigen and the virus is obscure. No more definite evidence has been brought forward for an association between viruses and these normal cell granules.

Viruses can be liberated from the cell structure both by fine grinding and by digestion with enzymes. Grinding is effective with tomato bushy stunt (4), tobacco mosaic (6), the tobacco necrosis viruses (5), and other plant viruses; but the duration and intensity of grinding needed to get satisfactory liberation are so great that they bring about other changes in the tissue. Not only is virus liberated, but it is also destroyed or becomes attached firmly to the fiber of the leaf. By this technique, therefore, no information is given about the manner in which virus is held by the tissue, and not very much about the total amount of virus that may be present. It is clear, however, that there is virus in the tissue apart from that present in solution in the cell fluids.

Digestion with trypsin sets free the microscopically visible Bollinger bodies from the fowl-pox-infected tissue in which they are imbedded, but it does not break the bodies down to the individual virus particles of which they are composed (28). Trypsin preparations also liberate vaccinia (27), tobacco mosaic (7), bushy stunt (4), and possibly poliomyelitis (17) viruses from infected tissues. The nature of the linkage that is being broken has in no case been ascertained; it is not even established that a protease, rather than some other constituent of the enzyme mixture used, is responsible for the effect.

It is obvious that enzymes capable of attacking cellulose and other polysaccharides are as likely as proteases to be able to liberate viruses from plant material. The digestive fluid from the crop of the snail (Helix aspersa) is a well-known source of cellulase, and it does in fact liberate both tobacco mosaic virus (7) and tomato bushy stunt virus (8) from infected leaves. With tobacco mosaic the residue after repeated incubation with trypsin still released abundant virus when ground finely or digested with snail enzyme, but efforts to release further virus after exhaustive incubation with snail enzyme were unsuccessful. With bushy stunt, on the other hand, trypsin will still release virus from the residue left after exhaustive incubation with snail enzyme. It may be, therefore, that the tobacco mosaic virus extraction is still incomplete and that a more assiduous search would lead to a technique for liberating still more. Already, however, the yield of virus accounts for one-third of the insoluble nitrogen of the leaf, and it is the infected leaf's principle nitrogenous component. Infection leads to some increase in the nitrogen content of the leaf, if this is expressed as a percentage of the dry matter, but the increase is insufficient to account for all the virus; in part, at least, this is replacing rather than supplementing normal protein. The leaves used in this work were not yet deformed or necrotic, and no experiments have been made to find whether an even larger proportion of the nitrogen would be present as virus if the duration of infection and the cultural condition of the plants were suitably controlled. It is a matter of very considerable interest that there can be so great a change in the type of protein present in the leaf without a more general interference with its working.

Exposure to the enzymes used in these experiments generally reduces or even destroys the infectivity of the liberated virus; but, with the viruses studied, there is no general destruction of the virus particles. These can therefore be recognized serologically or by isolation. Valuable information can be gained about the amount of material related to the virus that is present in the cell, but not about the distribution of infectivity among the particles that can, by their other properties, be characterized as virus, nor about the precise manner in which the virus particles are held. It is likely, however, that further work with purified enzymes will illuminate this problem by giving more examples of the phenomenon, already mentioned with tomato bushy stunt virus, of incomplete liberation by an enzyme that is destroying the morphological structure and subsequent enzymic liberation from the amorphous residue. It would be of the greatest interest to know whether this bound virus is still attached to the centers at which it was synthesized or whether it is

virus that has been in circulation in the cell and has become attached secondarily, but there is yet no evidence on this subject.

Virus attachment to the host cell has been more thoroughly studied when it is a preliminary than when it is a sequel to infection. The modification of the cell surface that is necessary to prevent fixation can be investigated either by stripping essential components from it or by covering them over by preliminary treatment with some other substance. The former technique was used by A. Pirie (25) in studying the adsorption of virus by *Bacillus megatherium*. She found that the bacterium could no longer adsorb virus after it had been incubated with egg white, and gave reasons for thinking that this change was due to the destruction of a polysaccharide on the cell surface by lysozyme. Similarly, Delbrück (15) finds that treatment with antibacterial serum greatly minimizes the adsorption of virus by *Escherichia coli*, and he suggests that the effect is due to a coating of antibody deposited on those regions of the bacterial surface that are involved in the attachment of the virus. The virus can likewise be made incapable of combining with the bacterium by previous combination with either bacterial polysaccharide (14) or antiviral serum (15). This last action is specific, and the serum has no effect on virus that has already been adsorbed by a bacterium.

There is little information about the mode of adsorption of influenza virus onto those tissues that are susceptible to infection, but there is much information about its adsorption onto red corpuscles. The virus is quickly adsorbed and then, after an interval that depends on the temperature and other conditions, released. The corpuscles, after this treatment, can no longer adsorb virus, but the virus can be adsorbed repeatedly onto fresh corpuscles. Hirst (19) points out the close resemblance between this process and an enzyme action in which a hypothetical enzyme on the virus destroys a substrate on the corpuscle surface to which the virus was attached. He brings forward evidence that the attachment of the invading virus to the lung surface is similar. In his view, the destruction of the receptor substance on the lung surface is a necessary precursor to infection. Nothing is known about the nature of the receptor substance nor about the effect of blocking agents on the process of fixation on either the lung or red corpuscle.

It is fairly certain that the phenomena described in the last two paragraphs occur at the surface of the host cell and that they involve the adsorption rather than the multiplication of the virus. There is some evidence about the processes going on inside the infected cell, but here there is less certainty and it is no longer possible to differentiate between agents that interfere with the attachment of the invading virus to the cell mechanism and agents that interfere with its reproduction on that mechanism.

Mutual antagonism of related viruses, when they are introduced at about the same time into a susceptible host, is well known in plants, animals, and bacteria. Luria and Delbrück (22) have found that an *Escherichia coli* virus, after such exposure to ultraviolet light that it will no longer cause infection, can interfere not only with the multiplication of a related virus strain but also with the multiplication of the same strain if this is introduced subsequently. The influenza viruses interfere with one another in the same way, and here also virus inactivated by ultraviolet light (29, 18) protects the chicken embryo from subsequent infection by active virus. It is difficult to picture any mechanism for this inhibition that does not involve competition between the active and inactive virus for a structure in the cell that is either rare or unique. This has been referred to as a "key enzyme" (22), and the protective action of inactivated virus is looked on as a case of the competitive inhibition familiar in the study of enzyme kinetics. There is no direct evidence on the nature of this attachment, but Fitzgerald and Lee (16) have published suggestive evidence that a substance related to ribose nucleic acid is involved either in the process of attachment or multiplication with a coli virus. Multiplication is specifically inhibited by 2-amino-9 (*p*-aminophenyl) acridinium chloride, and the inhibition can be reversed by ribose nucleic acid but not by a range of other substances. They point out that there is also chemotherapeutic evidence for a relationship between this and other acridines and the various metabolic cycles involving purines and pyrimidines. It is probable, however, that the acridine acts specifically on virus multiplication rather than generally on the energy exchanges of the cell, for acridine-resistant strains of coli could be developed and only those that were able to grow in the presence of this particular acridine had become insusceptible to the coli virus.

## CONCLUSION

In this survey attention has deliberately been directed as much to what is not, as to what is, known. In spite of this the picture is not unduly depressing. Although little is known definitely about the state of viruses in the infected cell, there are now many lines of approach from which the problem can be attacked. The histological approach is well known, and the visible structures seen in infected cells have properties that suggest that part of the virus is present in them in the form of a complex, for otherwise they would not stay out of solution. This interpretation depends on a knowledge of the intrinsic properties of the virus, and there is considerable evidence that these are not necessarily the same in the cell and in extracts. It is unquestionable, however, that a study of the properties of the viruses that have been purified imposes certain limitations

on the range of properties that it is legitimate to attribute to those viruses in the cell.

From a utilitarian point of view, the accumulation of evidence about the association of virus particles with one another and with other cell constituents is of importance, for the course followed by an infection may depend as much on these associations as on the rate at which virus can be synthesized in the cell. It is reasonable to suppose that some of the effects of the nutritional state of the host, and of the introduction of simple substances into the host, on the manifestations of a virus disease, may be due to the changing chemical environment affecting the state of attachment of the virus.

Finally—and this is perhaps the only factor that relates this paper to the main theme of the Symposium—the stability of a virus, and so presumably the likelihood that it will suffer variation or mutation, is bound to depend on the state of chemical combination in which it exists in the interval between its synthesis and the establishment of infection in another cell.

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## DISCUSSION

COHEN: Since Dr. Hotchkiss has raised the possibility of distinguishing the nonparasitic organisms from viruses on the basis of differing contents of metabolically active materials, especially phosphates, it is pertinent to present the data on this question.

All nonparasitic cells contain three types of nucleotides: (1) ribose nucleic acid (polymerized ri-

bose-3-phosphate nucleotides), found associated in the main with cytoplasmic particles—*e.g.*, chloroplasts, mitochondria, microsomes, etc.; (2) desoxyribose nucleic acid, found associated with some nuclear structures—*e.g.*, chromatin; (3) the ribose-5-phosphate nucleotides existing as various coenzymes, ATP, etc. Let us consider the distribution of these substances in the viruses.

All purified virus preparations have been found to contain nucleic acid. These include about a dozen plant viruses, which contain 5 to 40% ribose nucleic acid. All the phosphorus present in these viruses exists in the ribose nucleic acid.

Among the bacterial viruses, two, parasitic on *E. coli* B, have been purified and studied quantitatively with respect to their nucleic acid and phosphorus content. The T2 and T4 bacteriophages grown in *E. coli* in synthetic ammonium-lactate media contain 35-40% desoxyribose nucleic acid. The phosphorus to desoxyribose ratio of these viruses is 1:1. It has been claimed that some ribose nucleic acid is present in T2 grown on *E. coli* in nutrient broth and other media. Conceivably, preparations of T2 obtained from broth may be more difficult to purify. In any case, the absence of phospholipid has been noted by two groups of workers.

The rickettsiae of epidemic typhus, *R. prowazeki*, isolated from commercial egg-grown vaccines, have been found to have a small amount of desoxyribose nucleic acid. Ribose and ribose nucleotides were not found in rickettsial fractions.

Desoxyribose nucleic acid has been isolated from the elementary bodies of vaccinia and from the rabbit papilloma virus. Well over 90% of the phosphorus of these viruses is present in this nucleic acid. Ribose nucleic acid has not been found in either virus.

The PR8 strain of influenza virus has been reported to contain small amounts of both ribose and desoxyribose nucleic acid. This material contains antigenic groupings reactive with antisera to the normal cytoplasmic particles of the host from which the virus is derived. Hence it is difficult to conclude whether the small amount of ribose nucleic acid observed is an impurity derived from contaminating cytoplasmic granules, a portion of the cytoplasmic particles organically bound in the functional unit of stimulating virus groupings and host materials, or constitutes an intrinsic portion of the smallest grouping of structures capable of stimulating the production of influenza virus in a susceptible host.

Data on the nucleic acid content of some other materials are excluded from discussion since questions arise concerning (1) the purity of the preparation, (2) whether the material examined is really a virus, and (3) the techniques employed in the study of nucleic acid. In summarizing the data presented, it appears that among the viruses examined nucleic acid is present as either a ribose nucleic acid or a desoxyribose nucleic acid, and is rarely, if ever, present as more than one type.

Let us now consider the third class of nucleotides present in all nonparasitic cells. These are the ribose-5-phosphate nucleotides, present in the iso-alloxazine nucleotides, myoadenylic acid and adenosine triphosphate, and the di- and triphosphopyridine nucleotides, DPN and TPN, which act as coenzymes for a multiplicity of reactions involving the intermediary metabolism of phosphorus, fatty acids, carbohydrate, amino acids, oxidation-reductions, etc. The production and utilization of energy and cell substance of all nonparasitic cells are in large part dependent on the activities of ribose-5-phosphate nucleotides.

The close correlation between the phosphorus content of the viruses and their nucleic-acid content, whether of the ribose-3-phosphate type (ribose nucleic acid) or the desoxyribose phosphate type, although strongly suggestive of the absence of these phosphorylated coenzymes, cannot in itself exclude the latter substances, since the small amount of these materials would be undetectable by means of the relatively coarse phosphorus and sugar estimations. None the less, other evidence is pertinent to this question. Thus direct estimation of the flavin and nicotinamide content of several of the plant viruses and influenza virus has not revealed appreciable amounts of these coenzyme fragments. None of the viruses studied has been observed to possess an enzyme utilizing any of these coenzymes.

The presence of flavin adenine dinucleotide (FAD) has been reported in the elementary bodies of vaccinia. The relation of this substance to the virus is obscure, since the phosphatase activity of vaccinial preparations appears to be due to contamination with host materials. In any case, the limited utilizability of this coenzyme by this virus may be inferred from other data. Thus the cytochrome system and DPN and TPN appear to be absent within vaccinial elementary bodies, thereby eliminating some of the major transport systems with which FAD is linked in many metabolic reactions.

It would therefore appear that among the viruses examined the ribose-5-phosphate nucleotides are in general absent or limited in their utilizability. This deficiency in itself would determine in large part the dependence of the viruses upon a host for the production of energy and substance. At the present stage of knowledge concerning metabolism, one might say that an organism which had lost the use of its ribose-5-phosphate nucleotides would be unable to survive except as an obligate parasite.

Since many viruses lack important enzymes, which do not appear to require nucleotides in order to function, it would be incorrect to suggest that the two types of nucleotide deficiency completely describe the parasitism of the viruses. Nev-

ertheless, they do suggest important aspects of the parasitic relationship requiring study.

To summarize, in contrast to nonparasitic organisms which contain three types of nucleotides, each associated with important cellular structures and functions, the viruses appear to contain one type of nucleic acid, and are ill-equipped for the independent utilization of other nucleotides which may rarely be present.

KIDD: At the beginning of his paper, Dr. Pirie referred to the association, invariable and irreversible as studied thus far, between influenza virus and certain constituents of the tissue cells susceptible to its action, and also to the reversible association of mouse pneumonitis virus and components of its host cells. The literature of cancer provides two additional examples of complicated relationships between filtrable pathogenic agents and the components of cells susceptible to their action which may have interest in relation to the main theme of Dr. Pirie's paper.

Firstly, the filtrable agents responsible for sarcomas and related growths in fowls have long been known to have an intimate and complex relation with their host cells. Gye and his co-workers noted years ago that periods of months may sometimes pass during which the filtrable agent cannot be recovered from the sarcoma cells, though these continue to proliferate meanwhile, presumably as result of the activities of the associated pathogen, and after a further while the agent becomes readily extractable again. Aged normal fowls not infrequently develop substances in their sera that are capable of neutralizing the filtrable agents in the test tube, while neutralizing substances likewise appear in the blood of pheasants carrying a transplanted sarcoma that was originally induced in chickens with dibenzanthracene, as Andrewes found, though no filtrable agent could ever be got from the growth. Furthermore, Foulds has shown that antisera prepared in rabbits against a "nonfiltrable" induced sarcoma of chickens also possess the ability to neutralize the filtrable agent of Chicken Tumor I, and Amies has learned that sera produced in alien species against components of normal fowl tissues will neutralize the agents responsible for the Rous and Fujinami tumors, while attempts to dissociate the filtrable agents from the microsomes of fowl tissue cells by differential centrifugation have regularly been unsuccessful, as Claude and others know. Foulds, viewing all this, has concluded that an intracellular virus-protoplasm complex is formed in virus tumors, filtrability depending upon the dissociation of the complex in a particular way.

Secondly, the state of the Shope papilloma virus is very different in the epidermal cells of its natural host—the wild cottontail rabbit—than in those of domestic rabbits, though the latter are notably susceptible to its action. The papillomas in wild rabbits invariably yield large quantities of readily filtrable virus—unless this is masked by extravasated antibody, as not infrequently happens in the fissured confluent growths produced experimentally—and the free virus has a particle size somewhere in the neighborhood of 50 m$\mu$ and seems to be unassociated with normal tissue components. Domestic rabbit papillomas, by contrast, provide little or no free virus upon extraction, though there is abundant evidence that the virus persists in them and increases in amount as the papilloma cells proliferate. Comparative antigenicity experiments have shown that extracts of the papillomas of domestic rabbits are much less potent in stimulating the antiviral antibody than are extracts of cottontail papillomas, while furthermore whole extracts of the domestic rabbit growths must be employed if antigenicity is to be demonstrated, Berkefeld filtrates being devoid of this property. It is as though only a relatively small amount of virus were responsible for the continued proliferation of the papilloma cells, this being present in the cells of both species perhaps in roughly equivalent amounts and closely associated with formed cellular constituents, while those of the natural host, for reasons best known to themselves, produce a great excess of free virus in addition.

PIRIE: I am very grateful to Dr. Kidd for those comments because, although I was more or less aware of the phenomena he mentions, I could not have stated the position as clearly as he has done. My reason for omitting reference to them was an unwillingness to penetrate further than was strictly necessary into the perilous territory of tumor causation. For the sake of completeness, however, reference should also be made to Carr's observation (Carr, J. G., Brit. J. Exp. Path. 25: 56-62, 1944) that the serum from a chicken carrying a long-established or slow-growing Rous sarcoma would neutralize and precipitate extracts made from young tumors. He suggested that the nonfilterability of these old tumors, and of many other tumors, might be due to flocculation of the virus, in the extracts by antibody that would be simultaneously present.

# GENETIC SYSTEMS BASED ON HETEROCARYOSIS

## G. PONTECORVO

Genetics has been built on the study of species with sexual reproduction. An essential feature of genetic systems based on sexual reproduction is the alternation of meiosis and karyogamy, which has meaning only in relation to heterozygosity. These genetic systems could therefore be called "heterozygotic systems." For obvious reasons of economy, the mechanisms of heredity and variation in groups as important as the bacteria, the *Fungi imperfecti*, and others—in all of which sexual reproduction as defined above does not occur—have been disregarded by the geneticist. Yet as far back as 1926, when Muller wrote his far-reaching paper, "The gene as the basis of life," it was obvious that Mendelism was not the only way to a definition of the gene. Recently Beadle and Coonradt (*1*) with their work on Neurospora have given beautiful proof of what had been inferred from studies of somatic mutation in Drosophila, maize, etc.; namely, that even tests of allelism can be carried out without recourse to sexual reproduction. Pontecorvo and Gemmell (*8*) have confirmed this in a completely agamic species like *Penicillium notatum*, and other examples will be given here. The view that there cannot be a "genetics" of agamic species seems thus outdated. Classical genetics has taught us concepts and techniques which have some application in the study of any system of heredity and variation involving particulate units, even if not mechanically held together in chromosomes, provided these units have two essential properties of the gene: mutation, and reproduction in the mutated form.

The present paper attempts to describe certain genetic systems not based on karyogamy and meiosis but on segregation and recombination of whole nuclei in multinucleate cells. These systems seem to be universal in the *Fungi imperfecti,* widespread side by side with sexual reproduction in most other Fungi, and almost certainly occurring in other groups of microorganisms (Myxomycetes, Protozoa, Algae). To make their genetic study more attractive, there is also the likelihood that the mechanisms of heredity and variation in bacteria may become more easily approachable if we understand these. Certain Hyphomycetes, such as Penicillia and Aspergilli, will be taken as models, though the Ascomycetes, such as Neurospora, and the Basidiomycetes like the rusts and smuts—in which standard systems based on sexual reproduction coexist with these systems—have been and will continue to be extremely useful for the study of certain aspects of the problem.

The characteristics of the genetics of Hyphomycetes are as follows. (1) Sexual reproduction, involving fusion of nuclei and meiosis, is absent, or so rare as to play an unimportant part. (2) Most cells of a colony are multinucleate. (3) Pairs of cells occasionally fuse with each other. (4) Following such fusions, one or more nuclei may migrate from one cell into the other. If the nuclei of the cells that fuse are genetically different, one multinucleate cell, and part or all of its descendants, may come to carry nuclei that are not all alike. The same condition may, of course, arise as a consequence of mutation in one or more of the several nuclei of a cell. This condition is known as "heterocaryosis." Since ample reference to the pioneer work on heterocaryosis, mainly by Hansen, Dodge, and Lindegren, is given in a recent paper by Beadle and Coonradt (*1*), it will be dispensed with here. The terminology of heterocaryosis has been used in the most disconcerting ways; an attempt at unification is made in an Appendix at the end of this paper.

The analogy between heterozygosis and heterocaryosis is simple. In a heterozygous *nucleus*, allelomorphic genes are located in homologous chromosomes within that nucleus; in a heterocaryotic *cell*, the "allelomorphs" are located in different nuclei of that multinucleate cell. Segregation and recombination of the allelomorphs carried in a heterozygous nucleus require meiosis and karyogamy. Segregation and recombination of the "allelomorphs" carried in different nuclei of a heterocaryotic cell require an entirely different mechanism. Of course, in species like Neurospora where heterocaryosis and sexual reproduction coexist the standard mechanism of segregation and recombination may also operate, and in this case we may speak of allelomorphs without quotation marks. This is why species like these have been and will continue to be extremely useful: they bridge the gap between genetic systems of the usual types—"heterozygotic systems"—and genetic systems based exclusively on heterocaryosis—"heterocaryotic systems." In other words, they make it possible to attack with Mendelian techniques certain aspects of heterocaryosis. In exclusively heterocaryotic systems the absence of karyogamy and meiosis prevents the exchange of genes between nuclei. Thus differences between *nuclei* can arise only through mutation—in its broadest sense of change in quality, quantity, or arrangement of nuclear particles; but differences between *cells* can arise both through mutation and through recombination of genetically different nuclei.

The experimental study of heterocaryotic systems has barely started. Therefore the present paper can be no more than a statement of some of the fascinating problems they present and an

illustration of certain techniques by which these problems are being tackled. The comparative approach will be extensively used, because from the present knowledge of heterozygotic systems we can make useful predictions open to verification in heterocaryotic systems. Two main fields of comparative investigation are the genetics of metabolism (including growth and development) and the genetics of cell populations. In respect to the former we are faced with the action of genes carried in different nuclei of a cell; in respect to the latter we are faced with cells in which the assortment of nuclei can change in successive cell generations.

## Hyphal Fusions

In a growing colony of, for example, an Aspergillus or a Penicillium, hyphal anastomoses can readily be observed. They occur between branches of the same homo- or heterocaryotic hypha (Fig. 3) or between homo- or heterocaryotic hyphae of different origin (Fig. 4), which may or may not differ in the kinds of nuclei they carry. Inspection of a few slide cultures can leave no doubt that the frequency of hyphal fusions is very variable. The conditions controlling this frequency are, however, totally obscure. One factor is certainly the frequency of chance contacts between hyphae, which depends on the density of hyphae per unit volume of the medium. Thus if two colonies are grown on solid medium starting from inoculi far apart from each other, very few fusions will be observed when the colonies meet, because of the narrow "no-man's-land" between them which is crossed by only a few hyphae. On the other hand, if the two colonies are started close to each other, there is no gap between them, and fusions are abundant. Other factors affecting the frequency of hyphal fusions are the external conditions and, very probably, the genetic constitution of the hyphae that meet. With a number of strains—some X-ray mutants—of asexual species like *Penicillium notatum, Aspergillus oryzae,* and *A. niger,* and with the homothallic *A. nidulans,* interstrain fusions are so frequent as to ensure that if two conidia are planted a few tenths of a mm. apart on solid medium several fusions will be observable after a few hours' growth of the sporelings. Lindegren (7) has published some results that may indicate different frequencies of fusions in different combinations of strains of *P. notatum.* Clearly there is need of a simple technique for measuring the frequency of hyphal fusions in order to investigate all the various factors affecting it.

## Migration of Nuclei

That nuclei from one hypha can migrate into another, following hyphal fusions, has been shown again and again in many species of *Fungi imperfecti,* as well as in species having a sexual stage. When two homocaryotic strains which differ from each other in some detectable morphological or biochemical feature are inoculated side by side, nuclei of both kinds may be found together in the same cell in a larger or smaller proportion of the cells of the colony developing from the mixed inoculum. From a single hypha or conidiophore both original strains can be recovered following segregation of nuclei either via sexual reproduction or via one of the processes to be discussed later.

The details of such nuclear migrations, however, are totally obscure. We do not know, for instance, either how many nuclei migrate through each anastomosis or whether this number is constant or variable; nor in the latter case, do we know whether or not fusions without migration of nuclei may occur. In addition, we do not know whether migration is unidirectional or reciprocal at each point, or what is the influence of different combinations of strains on the direction of migration. Finally, we do not know whether nuclear migration occurs only at the moment of fusion or whether, so to speak, nuclei use the fusion to flow from one hypha into the other (and vice versa?) for quite a considerable time. One has only to glance at the mycological literature (*e.g., 13*) to realize what amazing things the nuclei of filamentous Fungi can do.

Clearly, such details are as essential for the investigation of heterocaryotic systems as the details of gametogenesis and fertilization are for the investigation of heterozygotic systems. Some of the techniques being developed for the study of nuclear ratios (*vide infra*) will probably be useful also in this respect.

## Multiplication and Segregation of Nuclei

Once a cell, or a hypha, has become heterocaryotic for two or more kinds of nuclei, what happens to the nuclei of the different kinds?

It is necessary first to stress the following points.

(1) The number of nuclei in cells of the same kind and in the same strain may vary very considerably, but is never very high; the higher numbers being of the order of tens. Cells of different kinds in the same strain (*e.g.,* conidia as compared with submerged hyphae) may also have quite different numbers. In *Penicillium notatum,* for instance, the submerged hyphae have up to a dozen nuclei per "cell," in *Aspergillus oryzae* the average is probably twice as much or more; the conidia are uninucleate in the former and multinucleate in the latter, but the nuclei are all derived from the same nucleus. In other species the conidia may be multinucleate, with the nuclei not all derived from the same one. In such cases the conidia may be heterocaryotic (*6*).

(2) The nuclei of the same "cell" divide independently of each other (Fig. 5). Contrary to what generally happens in syncytia and in multinucleate cells of higher organisms (*e.g.,* insect spermato-

gonia, pollen grains of Orchidaceae, megakaryocytes of mammals), the nuclei of multinucleate cells in the *Fungi imperfecti* are therefore not synchronized and may divide at different rates.

These two conditions—a variable but small number of nuclei per cell, and independent multiplication of each nucleus—make it conceivable that either or both of two mechanisms operate in determining the fate of the different kinds of nuclei in cells descendant from one that first became heterocaryotic. One assumption, made by most previous authors, is that the proportions of nuclei of different kinds in a cell are the consequence of random distribution at cell division, and that therefore nuclei of one kind may constitute, in different cells, anything from 0 to 100% of the total. The additional inference, drawn by Beadle and Coonradt (*1*), is that in the case of heterocaryons in which only certain definite proportions between the different nuclei endow the cell with maximal growth rate, those cells which, by chance assortment of nuclei, happen to have these proportions are favored by selection.

An alternative—or more probably a concomitant —mechanism, which has not been considered before, is that nuclei of different kinds may multiply at different rates and that these rates are in certain cases dependent on the proportions of the nuclei of different kinds in the same cell, or in a group of neighboring cells. Investigation of whether either or both of these mechanisms actually operate is at present in progress, the working hypothesis being that they both operate.

That random assortment at cell division does occur seems very probable. In the first place, there is the evidence from species forming multinucleate endogenous spores (*6, 3*). In these species some of the spores produced by a heterocaryon may be homocaryotic. In the second place, there is the evidence, reported below, from the comparison of heterocaryons that have a higher growth rate than either component homocaryon with heterocaryons that do not have this advantage.

The technique is as follows. By means of irradiation, "morphological" mutants are produced and two kinds of mutant are made use of: those with growth rates equal to the original strain (the "wild type"), and those with distinctively lower growth rates. To obtain mutants with growth rates equal to that of the wild type, the simplest way is to irradiate growing colonies of the wild type and isolate them as sectorial mutants. The shape of a sector (Figs. 1, 6, 7) gives an indication of the ratio of the growth rates of sector and mother colony (*cf. 9;* for mathematical treatment, *15*). On the other hand, to obtain "morphological" mutants with growth rates lower than that of the wild type, the simplest way is to irradiate spores, plate them out, and isolate colonies which show at the same time a suitable morphological change *and* a reduced growth

rate. Of course both types of mutant can also be obtained occasionally as a consequence of spontaneous mutation.

As first shown by Dodge (*3*), and confirmed by Beadle and Coonradt (*1*), pairs of morphological mutants often form heterocaryons morphologically different from either mutant but identical to the wild type, in the same way as in higher organisms a heterozygote for two nonallelic recessive genes may be different from either homozygous recessive but identical to the double-homozygous dominant. Thus, for example, a white-spored and a yellow-spored, or two different white-spored, X-ray mutants of *Penicillium notatum* form heterocaryons with green spores like those of the strain from which they were obtained (Fig. 8). Again, the two X-ray mutants of *Aspergillus oryzae* shown in Fig. 9—morphologically very different from wild type—form heterocaryons almost identical to the latter. Again, two light-colored "spontaneous" mutants of *A. niger* form heterocaryons with heads approaching the wild type in color (*4*). Many other examples could be given. In a culture started from a mixed inoculum of two such morphological mutants it is therefore possible to detect patches of heterocaryotic mycelium if they arise at all. Mutants differing from wild type in the color of the spore are particularly suitable in this respect.

The relevant fact is that from combinations of pairs of morphologically different strains two quite distinct patterns of heterocaryotic mycelium are obtained, according to whether the two strains both had growth rates lower than wild-type, or both—or at least one—had growth rates equal to the wild type. In the first case the heterocaryon very often grows faster than either component and may even grow as fast as the wild type. In a colony started from a mixed inoculum the heterocaryon soon overgrows the two homocaryons (Figs. 9, 11). It is in this respect similar to a "balanced" heterozygote, and the designation "balanced heterocaryon" seems appropriate. In the second case—when the growth rates of both, or at least one, strain are as high as that of the wild type—the heterocaryon is very unlikely to have a growth rate higher than that of wild type and therefore will not overgrow the two homocaryons, or at least not the one that has the wild-type growth rate. In this case a colony started from a mixed point-inoculum on solid medium has a striking appearance. At and around the point of inoculum there is a high proportion of heterocaryotic mycelium. But as the colony expands, it resolves itself into sectors of either component homocaryon, with only a few irregularly shaped strips of heterocaryon, which tend to vanish as they get farther from the center (Fig. 8). If inoculation has been made with a mixed-spore suspension, either on liquid medium or by flooding solid medium (Fig. 10), the pad appears as a mosaic of the two homocaryons dotted by patches of heterocaryon, variable

in size but generally small. Transplantation of one such heterocaryotic patch onto solid medium again produces a colony with a high proportion of heterocaryon at the center and decreasing proportions as the colony grows. A substantial heterocaryotic sector has never been observed in our heterocaryons. Thus a growth rate as high as that of the faster-growing component is not enough to enable a heterocaryon to establish itself as a lasting part of a growing colony. A plausible explanation of this is that the two kinds of nucleus are assorted at random at cell division and cells with nuclei all of one or all of the other kind arise from heterocaryotic cells faster than new heterocaryotic cells can arise from new fusions of homocaryotic ones.

This mechanism may also operate with "balanced heterocaryons," in which case, of course, if the heterocaryotic cells multiply at a rate sufficiently greater than that of either kind of homocaryotic cell, they can overcome the disadvantage of occasional extinction resulting from random segregation of nuclei. The physiological genetics of heterocaryons (*vide infra*) suggests, however, that selection in favor of heterocaryotic cells may not be the only process preserving the nuclear equilibria in balanced heterocaryons. An intracellular regulatory mechanism may conceivably exist, acting, for instance, through a differential effect on the rates of multiplication of nuclei of different kinds, whenever in a cell or group of cells their ratios have drifted away from the optimal range. In other words, selection of *nuclei* may operate as well as selection of *cells*.

Consider two "biochemical" mutants, A and B, differing from each other because A synthesizes metabolite alpha, but not beta, and B synthesizes beta but not alpha, both substances being diffusible and essential for growth. A will grow only on medium supplying beta, and B on medium supplying alpha; the heterocaryon, however, will grow on a medium supplying neither (*cf. 1* for many examples). Suppose that the synthesis of alpha and beta at optimal rates requires not less than a certain proportion of nuclei A and a certain other of nuclei B: optimal synthesis, and therefore optimal growth, including optimal rates of division of the nuclei, will take place in any cell or group of cells where the ratios of A to B nuclei lie within these optima. Suppose now that these limits are exceeded by chance, and a cell contains too high a proportion of B nuclei. In such a cell there will be a temporary abundance of substance beta and a shortage of substance alpha. Nuclei A, which can synthesize alpha, but require beta, will therefore be at an advantage and will multiply faster than nuclei B, thus re-establishing the equilibrium.

Techniques to test this hypothesis are being developed, and they may be useful also for elucidating other problems; for instance, that of how many nuclei migrate through each hyphal anastomosis.

They are based on the following reasoning. Experimental results (*vide infra*) support Beadle and Coonradt's (*1*) inference that in certain types of balanced heterocaryon the proportions between the two types of nucleus must be kept fairly constant during growth of the heterocaryotic mycelium. If the ratio for optimal growth were attained only as a consequence of chance assortment of nuclei at cell division, followed by a higher rate of multiplication of those cells that happen to have this ratio, a considerable number of cell divisions should be necessary on the average to produce one such cell after each anastomosis. If, on the other hand, it were attained as a consequence of differential multiplication of the two kinds of nucleus, it should be attained very rapidly, perhaps in the very cells that fused. The patterns of heterocaryotic mycelium obtained—*e.g.*, by flooding solid medium with mixed-spore suspensions—should be different in the two cases, and from their study it might be possible to deduce which is the process actually involved. Several variables can be experimentally controlled: (1) the medium, which probably determines different optimal nuclear ratios; (2) the proportions between spores of the two kinds; and (3) the density of the spore suspension, and therefore the frequency of chance contacts per unit area. These techniques are still in a very tentative state, and I shall therefore refrain from further speculation.

### Physiological Genetics of Heterocaryons

It has been pointed out by most previous authors that, from certain points of view, the physiological effects of genes carried in one nucleus, as we know them from classical genetics, are paralleled by those of genes carried in the different nuclei of a heterocaryotic cell. We may thus have dominance in a heterocaryon, just as in a heterozygote, though in the former the "alleles" are carried in different nuclei of a multinucleate cell and in the latter they are carried in the same nucleus.

In two important respects, however, we should expect the physiological genetics of heterocaryons to differ substantially from that of heterozygotes. The first is that in a heterocaryon the different kinds of nucleus can be represented in different proportions from one cell to another, and there is some evidence that these proportions may be controlled, at least selectively, by conditions outside the cell. The second is that, in the case of genes whose action is affected by intranuclear conditions (*e.g.*, position effects), or of genes with localized intranuclear action (*e.g.*, genes affecting specifically the mutation rates of other genes), the parallel between heterocaryons and heterozygotes breaks down.

While dominance in heterocaryons has been amply demonstrated, "autonomous" and "nonautonomous" action of the genes has not hitherto been
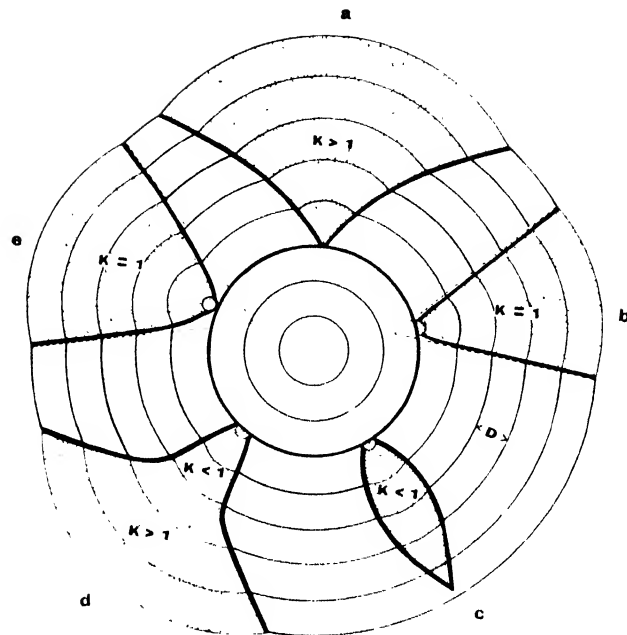
Fig. 1. The shape of a "sector" in a colony gives an indication of the ratio (K) of the growth rate of the sector to that of the mother colony. Various types of sector obtained after irradiation of a growing colony are shown. The heavy circle indicates the growing edge of the colony at the time of irradiation. (Reproduced from Nature 154.)
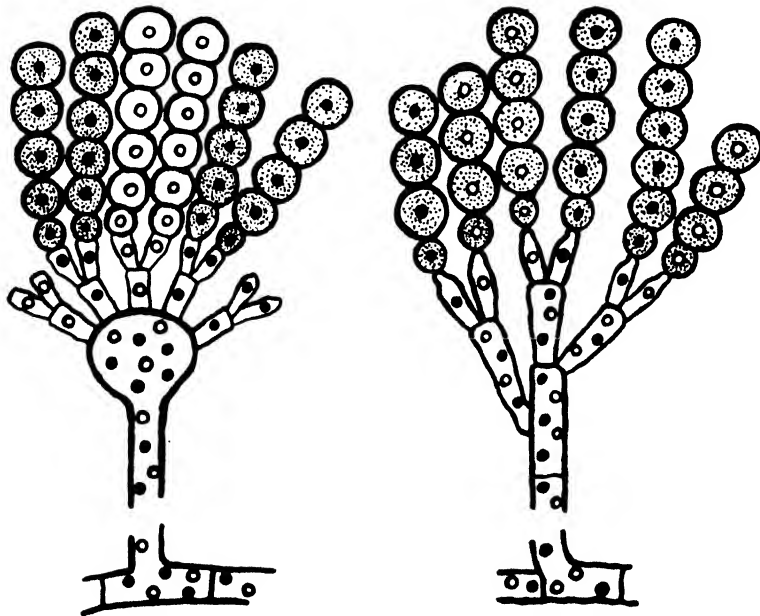


Fig. 2. "Autonomous" (*left*) and "nonautonomous" (*right*) nuclear action in heterocaryons. Left, the pigment of the uninucleate conidia is determined by the kind of nucleus segregated in each conidium. Right, the pigment is determined by the heterocaryotic conidiophore and not by the kind of nucleus segregated in each conidium.
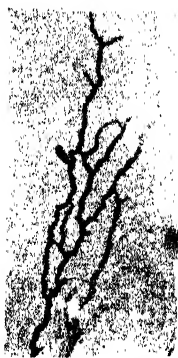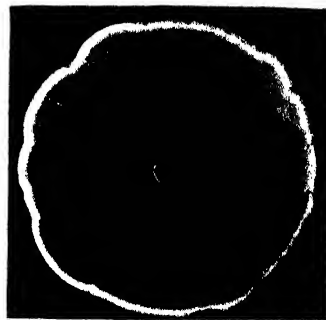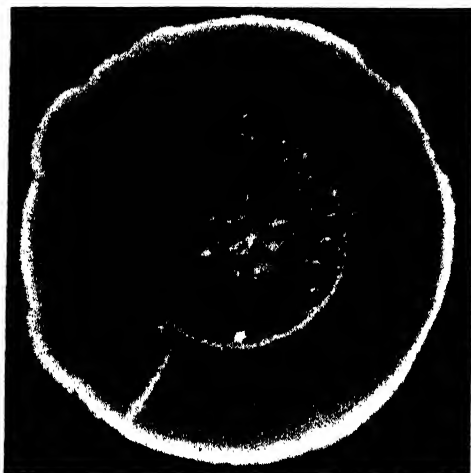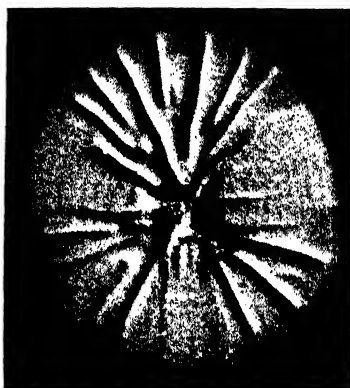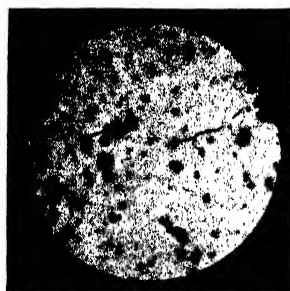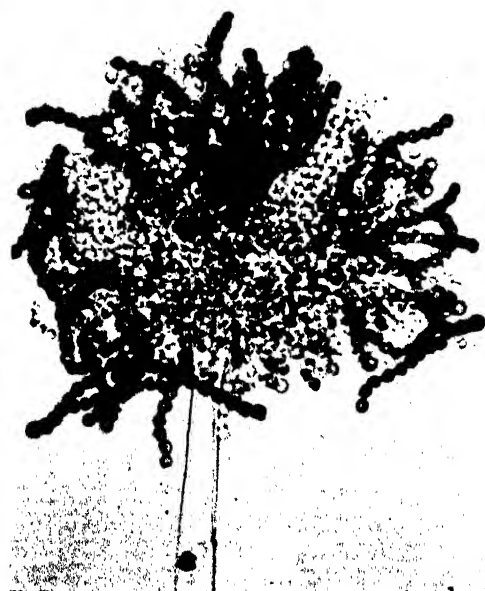
FIGS. 3-14 (see opposite page for legends).

shown to have its counterpart in them. This can now be demonstrated.

In species like Aspergilli and Penicillia, most of which produce conidia with one nucleus, formation of conidia in a heterocaryon automatically leads to segregation of nuclei of different kinds. This has been shown to be the case for both balanced and nonbalanced heterocaryons, in *Aspergillus niger* and *A. nidulans* (4), in *A. oryzae* (present paper), and in *Penicillium notatum* (8); and it holds also for microconidia in Neurospora (11).

The fact that conidia are, in these species, uninucleate, or at least (e.g., in *A. oryzae*) carry nuclei all derived from one nucleus, makes it possible to investigate whether the action of certain genes is cell-localized or otherwise. Consider two color mutants; e.g., a yellow-spored and a white-spored, both obtained from a green-spored wild type. The heterocaryon between these two mutants will carry both "white" *and* "yellow" nuclei in its hyphae and conidiophores but will allot *either* a "yellow" *or* a "white" nucleus to each conidium. What color will each conidium develop? From classical genetics we would expect quite different results in different cases. If the "yellow" and "white" were recessive and nonallelomorphic, their dominant alleles being necessary for the production of two diffusible substances, we should expect the color of every conidium to be green, irrespective of which kind of nucleus was segregated into it ("nonautonomous gene action"). If, on the other hand, the two dominant alleles were necessary for the normal performance of two metabolic steps strictly localized within the conidium, we should expect the conodia to be *either* yellow *or* white (never green) according to which

nucleus they received ("autonomous gene action"). The various other possible combinations of "autonomy" and "dominance" can be easily worked out, and there is no reason to doubt that they may all occur in heterocaryons.

So far, examples of both extreme types of action, autonomous and nonautonomous, with dominance have been found; they are diagrammatically represented in Fig. 2. An example of nonautonomous action is given by heterocaryons between two white-spored X-ray mutants of *Penicillium notatum*, and between each of them and a yellow-spored mutant. The conidia produced by such heterocaryons are all green, like those of the wild type, though they give origin to either white or yellow colonies (8). The same situation has been described by Gossop, Yuill, and Yuill (4) for heterocaryons between pairs of color mutants of *Aspergillus niger*.

I am indebted to J. L. and E. Yuill for having called my attention to two examples of the other kind of gene action (autonomous) and for having allowed me to use their remarkable photographs. Heterocaryons between *A. nidulans* (green spores) and its white-spored mutant "alba" produce heads in which single chains of conidia are either green or white (Fig. 13). Heterocaryons between a green-spored and a light-colored mutant of *A. tamarii* again produce heads with either green or light-colored chains (Fig. 14). Incidentally, this constitutes a beautiful genetical proof that the conidia in a chain all have nuclei of one kind, presumably derived by repeated division of the nucleus in the sterigma.

As a last remark on the physiological genetics of heterocaryons, it is necessary to stress that in

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LEGENDS FOR FIGURES 3-14 (see opposite page)

FIGS. 3 and 4. Hyphal anastomoses between (3) branches of the same hypha (*Aspergillus oryzae*, X-ray mutant 2B) and (4) different hyphae (*A. nidulans*).

FIG. 5. *Penicillium notatum*, showing several nuclei per "cell" dividing independently. X 800, Zirkle's fixing-staining-mounting carmine.

FIGS. 6 and 7. Mutant sectors of various shapes arising after irradiation of growing colonies of *P. notatum*; the sectors originate from the growing edge of the colony at the time of irradiation. The strain isolated from the narrow white sector in 7 has a growth rate identical to that of the parent strain. (Fig. 6 reproduced from Nature 154.)

FIG. 8. Colony arising from mixed point-inoculum on solid medium of two white-spored X-ray mutants of *P. notatum*. Heterocaryotic patches with green spores, like the wild type, originate at and around the position of inoculation but fade away as the colony grows. Both mutants have growth rates equal to that of the wild type, and the heterocaryon has no greater rate.

FIG. 9. *Top*, *A. oryzae* (wild type); *left*, X-ray mutant 1; *right*, X-ray mutant 2B; *bottom*, heterocaryon produced by inoculating mutants 1 and 2B together. All started by point-inoculum at the same time. The heterocaryon has a growth rate almost equal to wild type and much greater than either mutant.

FIGS. 10 and 11. The same pairs of mutants as in 8 and 9, respectively, but inoculated by flooding a surface with a thick mixed-spore suspension. The heterocaryotic patches in 10 (darker) arise all over the surface but cannot compete with the two homocaryons; on the other hand the heterocaryotic patches in 11 (fluffy) are rapidly overgrowing the homocaryons.

FIG. 12. Segregation of the two kinds of nucleus in the heterocaryon between *A. oryzae* mutants 1 and 2B. Plating of conidia produces colonies of type 1 or 2B. Occasional heterocaryotic colonies arise through new fusions between sporelings lying close to each other on the medium.

FIGS. 13 and 14 (courtesy of J. L. and E. Yuill). "Autonomous" nuclear action in heterocaryons. Heterocaryotic head of *A. nidulans* (green spores) and its mutant *alba* (white spores), showing chains of green and white spores respectively (13). Similar situation with color mutants of *A. tamarii* (14).

most Fungi there seems to be a continuity of cytoplasm between cells in a hypha, and it is even possible that nuclei can migrate from one cell to the next. In addition, the cytoplasm is very actively streaming but apparently the nuclei are not carried in the flow (13). All of these details are still controversial. A provisional picture is that of a hypha in which the products of the activity of the various nuclei are readily mixed so that we may perhaps consider it as a single unit. If this picture is broadly correct, we may usefully treat the nuclei within a hypha as constituting a single population.

## CONTROL OF NUCLEAR RATIOS

It was shown by Dodge (3) and amply confirmed by Beadle and Coonradt (1) that when two mutant homocaryotic strains have lower growth rates than the wild type, the heterocaryon between them may have a higher growth rate than either mutant and often as high as that of the wild type. Thus in a growing colony started from mixed inoculum the heterocaryon readily supplants both homocaryons and the heterocaryotic condition is preserved by selection, either of heterocaryotic hyphae or, as put forward here, of nuclei within a hypha—or, more probably, of both. In their remarkable theory of balanced heterocaryons, Beadle and Coonradt assume that in those made up of two components there will be a range of ratios between the numbers of nuclei of each kind within which growth will be maximal, and that selection will keep the ratios within this range. How large this range will be depends on the dominance relations of the two or more pairs of "allelomorphs" involved. To simplify: if nuclei of strain 1 are (aB) and those of strain 2 are (Ab), the heterocaryon 1 + 2 will have (aB) (Ab) (capital letters indicate dominants, and parentheses indicate different nuclei). If both A and B are strongly dominant—i.e., if the metabolic step controlled by each of them is performed in a cell at full rate even if nuclei carrying A (or B) constitute 50% or less of the total—then there may be a wide range of nuclear ratios compatible with a growth rate as high as that of the wild type ("haplo-sufficient" genes of classical genetics). If, on the other hand, both A and B are weakly dominant—i.e., if 50% or more nuclei carrying A (or B) are necessary for full performance of the metabolic step controlled by A (or B),—then a growth rate as high as that of the wild type may never be attained by the heterocaryon, and the maximal growth rate will be compatible with only a narrow range of nuclear ratios.

Beadle and Coonradt, as well as Sansome (12), have sampled the nuclear constitutions of a number of two-component balanced heterocaryons of *Neurospora crassa*. In duplicate tests of three heterocaryons having growth rates as high as the wild type, the ratios of nuclei of one mutant to those of the other were found by the former authors to vary

as follows: 1:17.6 and 1:1.6; 1:8 and 1:1.5; 1:2.2 and 5:1. Even though the technique of sampling has many pitfalls, there is no doubt that in these cases the nuclear ratios varied over a wide range, as expected on the basis of the theory. On the other hand, one of our balanced heterocaryons between two X-ray mutant strains of *A. oryzae* seems to satisfy the expectation of the theory for the alternative situation. The heterocaryon (Fig. 9) has a growth rate slightly lower than that of the wild type but much higher than that of either component (strains 1 and 2B). Four heterocaryotic cultures grown at the same time and sampled by plating spore suspensions gave the following ratios of nuclei 1 to 2B: 1:2.7 (130/347); 1:2.9 (483/1388); 1:3.1 (251/777); 1:2.6 (574/1485)—the numbers between parentheses indicating the numbers of colonies in each sample (heterocaryotic colonies were counted as two, one of each kind, since in the majority of cases it is possible to see that they originated from new fusions between sporelings of the two types lying side by side, Fig. 12). It is remarkable that in nonsystematic samplings made earlier the same two strains had consistently given nuclear ratios of about 1:6. Obviously, suggestive as these results are, more work—especially with strictly controlled external conditions—will be necessary before we can consider it as proved that nuclear ratios are selectively controlled (*vide infra*).

An amplification of the theory of balanced heterocaryons is immediately apparent if we consider balanced heterocaryons made up of more than two components. I am indebted to my colleague J. G. Carr for having pointed it out to me. In the case of two kinds of nuclei, carrying respectively the weak dominants A and B, no ratio of nuclei (Ab) to (aB) will give full growth, for the simple reason that it is impossible to have in the same cell more than 50% of nuclei (Ab) as well as more than 50% of nuclei (aB). But if we have three kinds of nuclei, (AbC), (aBC), and (ABc), forming, two by two, heterocaryons which for the same reason as before do not show full growth, their three-component heterocaryon may well show full growth. In fact, in such a heterocaryon the A, B, and C "alleles" can each be present in proportions up to 66.6%. A fortiori, a four-component heterocaryon, (AbCD) + (aBCD) + (ABcD) + (ABCd), may show full growth while combinations two by two or three by three of the component strains fail to do so; in this case the A, B, C, and D "alleles" may each be present in proportions up to 75%. Suitable mutant strains for testing these deductions are being prepared. Should the preceding reasoning be correct, the conclusion should be drawn that multiple-component balanced heterocaryons have greater adaptability than those made up of only two components.

So far we have considered nuclear ratios as adaptively regulated on account of the genetical proper-

ties of the nuclei themselves. In other words, if an essential metabolite alpha, necessary for growth, is produced at a certain rate under the control of nuclei *A,* and another metabolite beta under the control of nuclei *B,* the ratio of nuclei *A* to *B* in a heterocaryon will be regulated in such a way as to give the outputs of alpha and beta that result in maximum growth. However, if alpha and/or beta were diffusible substances which the colony could also obtain from the medium, the adaptive ratios of the two kinds of nuclei would be expected to vary according to the amounts of either or both substances available in the medium. Heterocaryotic systems may thus present us with a novel type of adaptation to external conditions: an adaptation which may even be based on an intracellular regulation of the genotype. Clearly we are not far from some kind of Weissmanian segregation of determiners during growth and differentiation, and its theoretical implications are obvious enough.[1] This line of investigation may also link up with the study of bacterial variation in response to environmental conditions.

### POPULATION GENETICS OF HETEROCARYOTIC SYSTEMS

It would be futile to embark on a detailed genetical theory of populations in heterocaryotic systems before at least some of the processes discussed so far are more precisely understood. It may be useful, however, to consider the distinctive features of some genetical situations in such populations.

In the first place, as already pointed out, we may be justified in considering a hypha as a mass of cytoplasm with a population of nuclei. Such a population is subject to: (1) variation in numbers; (2) drift—i.e., random variation in the proportions of the different kinds of nucleus; (3) migration—i.e., influx and outflow of nuclei, following hyphal anastomoses; (4) mutation; and (5) selection. Selection may act either on the nuclei themselves as proposed here, or on the hyphae carrying them. There are here all the elements considered by Fisher, Haldane, and Sewall Wright in their work on the genetical theory of populations in the case of heterozygotic systems. No doubt their techniques will be to a large extent adaptable to, and extremely useful in, the study of heterocaryotic systems when the time has come for a parallel treatment. If the balancing of nuclear ratios is actually based on a mechanism like that proposed, then the cytoplasmic milieu, which must determine the way in which selection of nuclei works, must also in its turn depend on both the nuclear ratios and the extracellu-

lar conditions. The mathematical handling of such a situation is probably difficult; but, in comparison with heterozygotic systems, the possibilities of obtaining quantitative experimental data are here considerably greater. For instance, we may vary the nuclear ratios by combining different strains—two by two, or three by three—and we may vary the external conditions quantitatively by supplying in various amounts one or more of the substances that these strains are unable to synthesize.

One of the problems requiring elucidation is that of whether and how heterocaryosis is perpetuated in nature. We know that it can be perpetuated in those species that form multinucleate conidia, since the conidia may contain nuclei of different kinds. But we know nothing about species with uninucleate conidia, in which formation of the conidia inevitably leads to segregation of the different kinds of nucleus. There is, of course, the possibility that in these species a high proportion of airborne conidia may be dispersed in groups rather than singly. The alternative would be that reconstitution of heterocaryons is left to chance side-by-side germination of conidia with different kinds of nucleus. It would be extremely useful to expand researches on the mechanics of spore dispersion, like those reviewed by Gregory (*5*), so as to include the study of these problems.

### VARIATION IN BACTERIA AND HETEROCARYOSIS

Since the present paper is largely speculative, a little more speculation may be pardoned. Can the study of heterocaryosis help towards the understanding of certain aspects of variations in bacteria? Sectoring, secondary colony formation, mass changes in response to the medium, mutation, etc., are all features common to variation in bacteria and *Fungi imperfecti.* On the other hand, most bacteria have no visible nuclei. Since the results of irradiation suggest that they have a particulate type of inheritance (*cf. 14, 10, 2*), each kind of particle could be represented in each bacterium in many replicas, to ensure that it is handed over at cell division despite the absence (?) of a precise mechanism like that of mitosis. It may also be assumed that, similarly to the intracellular regulation of nuclear ratios, postulated in heterocaryons, the different kinds of particle are kept in equilibrium in each cell by differential multiplication, but are also liable to random variation in frequencies at cell division, including occasional complete loss. If, in addition, particles can be exchanged between two bacteria, the whole picture should be similar to that of heterocaryotic systems with the difference that in Fungi the units of segregation and recombination are whole nuclei while in bacteria they might be of a more elementary nature, say genes or groups of genes (*2*).

The present hypothesis could be tested (as suggested to me by Dr. S. E. Luria) by the technique

[1] Since the above was written two important papers have appeared. Ryan and Lederberg (Proc. Nat. Acad. Sci. 32: 165-173, 1946) give a definite proof that external conditions may shift the nuclear ratios in heterocaryons. Lederberg (Science 104: 428, 1946) makes a stimulating suggestion on the uncontrolled growth of cancer tissues.

already described for Fungi; i.e., mixed inoculation of pairs of morphological mutants, and search for patches of nonmutant growth. Single cells of this type should segregate for the two components. (Part of this hypothesis is supported by the results of Lederberg reported at this Symposium.)

*Note added in proof:* The demonstration (Demerec and Latarjet, this Symposium) of the one-hit type of dependence of mutation on dosage of irradiation in *E. coli*, and the finding (McIlwain, Nature 158: 898-902, 1946) that certain enzymes may be represented by only one or very few molecules per cell in bacteria, are against the hypothesis of each kind of particle being present in a variable but sufficiently high number of replicas. A more likely alternative, pointed out to me by Dr. H. J. Muller, is that the hereditary particles of different kinds in bacteria—and perhaps in viruses as well, in view of Delbrück's and Hershey's results (this Symposium) —are (linearly?) kept together in a way basically similar to that occurring in higher organisms.

### CONCLUSIONS

Clearly, heterocaryotic systems call for a novel type of genetics; though, of course, the body of knowledge of classical genetics will make the task enormously easier than would otherwise have been the case. The *Fungi imperfecti* are probably the most suitable material for starting these investigations, with the additional precious help of species like Neurospora where heterocaryosis and sexual reproduction coexist. The essential feature of heterocaryotic systems is that they are based on mechanisms of segregation and recombination of hereditary particles other than meiosis and karyogamy. In this respect, the choice of the *Fungi imperfecti*, with their apparently normal types of nucleus, should not close our eyes to the implications of these studies for the attack on heredity and variation in other important groups, particularly the bacteria.

### APPENDIX ON TERMINOLOGY

*Nucleus* with one or more sets of homologous chromosomes: haploid, di-, tri-, tetra-, polyploid.
*Cell* with one or more nuclei: uni-, bi-, tri-, quadri-, multinucleate.
*Tissue, Colony, Individual* with one or more cells: uni-, bi-, etc., multicellular.
*Nucleus,* di-, or polyploid, carrying genetically and/or structurally identical (or different) homologous chromosomes: homozygous, heterozygous.
*Cell,* bi-, or multinucleate, carrying genetically and/or structurally identical (or different) nuclei: homocaryotic, heterocaryotic.
When there is no danger of confusion, attributes of one structure may be used for a higher structure. For example, a homocaryotic colony is one made up of homocaryotic cells; a heterozygote is a multi-

cellular individual with all (or, better, practically all) cells heterozygous, etc. The author favors spelling heterocaryosis with a k (heterokaryosis).

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### DISCUSSION

LEDERBERG: Experiments with heterocaryons in Neurospora bear out most of Dr. Pontecorvo's conclusions. Neurospora heterocaryons, however, seem to be more stable than those in Penicillium or Aspergillus, even where there seems to be no selective advantage in heterocaryosis. Heterocaryons between wild type and most biochemical mutants do not in general segregate out in presence or absence of the growth-factor requirement of the mutant. Heterocaryons involving nuclei carrying two distinct albino genes show pigmented conidia through many transfers, and on plates, with no evidence of spontaneous segregation. By isolating hyphal tips, one occasionally does secure a homocaryotic culture, as evidenced by lack of pigment. This is a rare occurrence, however.

Combinations between two Neurospora species (*N. crassa* and *N. sitophila*) may exhibit a different behavior. An albino-p.a.b.-less crassa (1633-15300) and a pyridoxinless sitophila (299) will grow together on minimal medium. The components will segregate out very readily according to whether p.a.b. or pyridoxin is supplied. Even on minimal medium in plates, one finds patches of albino conidia, suggesting a segregation of the crassa nuclei.

WHITE: It seems to me that the analysis of heterocaryotic systems would be greatly facilitated if one could obtain heterocaryons containing visibly different types of nucleus. Since there is no meiosis in the forms you have been working on, I suppose that one can consider their nuclei to be essentially haploid. But it is presumably possible to obtain polyploid nuclei by the use of colchicine, camphor, or some other substance. Would it not be worth while to make heterocaryons containing nuclei of different degrees of ploidy, which would presumably be of visibly different sizes, so that one could determine what happens during hyphal fusion and other phases in the cytology of the *Fungi imperfecti*?

PONTECORVO: This is a very good suggestion, and has indeed already been considered. Mrs. Sansome has produced by camphor treatment giant conidia in *P. notatum;* but, as far as I know, it is not yet clear whether the nuclei are distinguishable in size from those of an ordinary strain. The nuclei of these fungi are so small and so erratic in their staining reactions that even an apparently simple comparison between nuclei differing in their volume by a factor of two or four may be a difficult proposition.

# PLASTID MUTATIONS

## M. M. RHOADES

At the present time it is commonly believed that the plastid is an independent self-reproducing entity capable of undergoing mutation. The basic unit of the plastid, which has been called the plastogene (*18*), and which may be at certain stages of no more than macromolecular size, belongs with those protoplasmic elements—gene, plasmagene, and virus particle—which are able to duplicate themselves. That genes, plastids, and viruses all contain or consist of nucleoproteins is of more than passing interest in this connection.

The belief that plastids of higher plants are autonomous rests chiefly upon the non-Mendelian heredity of certain plastid variegations, where it appears that two distinct and separable kinds of plastid are involved. Inasmuch as the two plastid types are assumed to have arisen by mutation, it is evident that the study of plastid variegation is of fundamental importance to the question of the plastogene as an independent hereditary unit.

Chlorophyll variegations are believed to arise in the following ways: (1) Two distinct types of plastid are present in the cytoplasm of the fertilized egg, and these undergo somatic segregation. (2) One type of plastid is present, but changes in plastid phenotype occur during ontogeny. These may be due to (a) plastid mutations, either gene-induced or spontaneous (in the sense that they are not caused by any known nuclear gene), or to (b) varying degrees of plastid development in response to the different physiological conditions found in diverse tissues. These different metabolic conditions may be gene-controlled (pattern genes) or caused by some infective agent. In either case no mutation of the plastogene is involved. Classes 1 and 2a require the presence of distinct plastid types, which are derived by mutation, and it is the purpose of this paper to examine the evidence for plastid mutation. No attempt will be made to review the extensive literature on inheritance of plastid differences, as this has been done in a comprehensive manner by Correns (*8*) and Renner (*26*).

### HYPOTHESIS OF SOMATIC SEGREGATION, OF MIXED PLASTIDS

A large number of cases of non-Mendelian heredity of plastid differences (variegation or striping), where the transmission is not influenced by nuclear genes, have been described. The most common of these is that in which the plastid variegation is inherited through the mother only—i.e., uniparental. Correns (*5*) first described this type of heredity in *Mirabilis jalapa*. This and similar cases have been designated as "status albomaculatus,"

and the breeding behavior of this type of plastid variegation is as follows: (1) inheritance is through the mother only, with the pollen having no effect on the character of the progeny; (2) flowers on green branches give only green offspring, white (palegreen or yellow) branches yield white progeny, while variegated branches produce green, white, and variegated individuals in widely varying ratios.

At the present time a number of interpretations are held to account for this mode of inheritance. The simplest and most widely accepted view is that the variegated plants possess two distinct kinds of plastids (green and white). It is assumed that the white type arose through mutation and that the two types become separated by somatic segregation during cell division. Baur, Gregory, Winge, Chittenden, Imai, and many others hold to this explanation. The hypothesis of somatic segregation of mixed plastids demands a genetic autonomy and independence of the plastid types. The breeding behavior is seemingly in harmony with the hypothesis, but cytological observations yield discordant results. Gregory (*13*) found two types of plastids in *Primula sinensis albomaculata*. The mature green portions of variegated plants had only normal plastids and the chlorotic areas had only smaller, nearly colorless plastids, while both types were observed in embryonic cells. Similar observations on different plants have been reported by other investigators. Here then appears to be the cytological confirmation needed to complement the genetic data. Randolph (*24*) doubts the correctness of these observations, since he found in maize that embryonic cells of normal plants have plastids in various stages of enlargement, and suggests that this range in size has been mistakenly interpreted as indicating the presence of two types of plastid.

In maize four cases of maternally inherited chlorophyll variegation have been described; the two most detailed accounts are by Anderson (*1*) and Demerec (*10*). Anderson's and Demerec's maternally inherited chlorophyll striping duplicates in all respects the type of behavior observed by Correns in Mirabilis, by Baur in Antirrhinum, by Gregory in Primula, and in other classical examples of "status albomaculatus." Moreover, both Anderson and Demerec made the important observation that the three classes of offspring (green, striped, and yellowish) were not distributed at random on the ear. There was a definite grouping or clustering of each class on the ear, with the striped seedlings occurring in a transitional region between the green and yellow zones. A similar nonrandom grouping was found by Karper (*19*) in sorghum. This non-

random clustering of the three seedling types suggests the somatic segregation of some cytoplasmic component, and on the basis of the mixed-plastid hypothesis this would be the plastids. Randolph (*24*), however, studied plastid development and morphology in the maternal strain described by Anderson. He did not find two distinct types of plastid in the embryonic cells of striped plants, although the plastids in pure yellow cells were usually smaller, paler, and less numerous than those in green cells. Some plastids in pure yellow plants had a diameter and a green color equal to that of plastids in normal green cells. The transitional region between green and yellow areas on striped plants contained plastids showing all degrees of variation found in either pure yellow or green areas. Moreover, the boundary between green and yellow stripes was never sharply delimited; always there was a region of transition in which were found even within a single cell plastids showing many intermediate sizes and depths of color. In some instances the transitional region was one cell wide, while in others it was several cells wide with each cell having all or nearly all of the plastids with intermediate shades of color. Correns (*5*) found a similar gradation in color intensity in those cells that formed the boundary between green and whitish areas in Mirabilis. Randolph's and Correns's observations cannot be reconciled with the hypothesis of mixed-plastid segregation. Baur, who proposed the hypothesis of the segregation of mixed plastids to explain the variegation found in Pelargonium, came to the conclusion in 1930 that in Mirabilis a defective cytoplasm rather than the plastids was the cause of the variegation.

Correns (*5*) believed that a diseased cytoplasm was responsible for the variegation of albomaculata plants, but later (*6*) adopted the view that the cytoplasm in embryonic cells of variegated plants exists in an indifferent labile condition, which changes fortuitously into a normal state that permits plastid development or into a diseased condition that prevents normal plastid development. He could not accept the hypothesis of plastid segregation because of the discrepancy between the number of cell divisions required for a complete separation of two plastid types and the number of mitoses needed for embryo development. Another weighty objection was the virtual absence of cells with two types of plastids, although cells with mixed-plastid types should be numerous since the descendants from an embryonic cell with two kinds of plastid would have a far greater chance of possessing plastids of both types than of being pure for either kind.

East (*11*) thought it probable that an active, parasitic agent present in the cytoplasm is responsible for cytoplasmically inherited chlorophyll variegation.

I agree with Correns that some cytoplasmic condition other than the plastids furnishes the correct explanation for the type of inheritance found in "status albomaculatus." I believe, however, that the observation of Anderson and Demerec that in maize the green, striped, and yellow seedlings are not distributed at random on the ear forces us to assume that the somatic segregation of some discrete cytoplasmic component controlling plastid development does occur. Whether or not we designate this hypothetical entity as a plasmagene is a matter of choice. Two alternatives are possible. We may be concerned with a plasmagene affecting plastid development that exists in both a normal and an abnormal form; or we may be dealing with one kind of plasmagene, which is not present in sufficient number to insure both daughter cells always receiving enough of them for normal plastid growth. On the basis of the first alternative, the ratio of abnormal to normal plasmagenes would determine the type of plastid development. Those cells with a critical percentage of abnormal plasmagenes would contain poorly developed plastids, while those cells with higher proportions of normal plasmagenes would have more normally developed plastids. The occurrence in Anderson's strain of a transition zone —with graded, intermediate plastid colors—between green and yellow stripes that have different cell lineages, suggests that these plasmagenes are able to pass through cell membranes. It also argues in favor of the first alternative, which we shall adopt. This hypothesis differs from Correns's in that the causal agent of the striping (or variegation) is believed to be a discrete molecular entity in the cytoplasm, while Correns apparently considered the cytoplasm to be a homogeneous mass. The genetic data make it necessary to endow this hypothetical plasmagene with the power of autocatalysis. This hypothesis, which assumes the somatic segregation of two kinds of plasmagenes, is confronted with the objection raised by Correns against the mixed-plastid hypothesis—namely, the large number of cell divisions needed for a final separation into pure types. It is not necessary to assume, however, that a colorless cell has only one type of plasmagene; it may need only a preponderance of the abnormal type.

In summary, there is no convincing evidence that the type of variegation known as "status albomaculatus" is due to the existence of distinct plastid types. Consequently, the analysis of this type of plastid variegation throws no light on the questions of plastid mutation and plastid autonomy.

The behavior of variegation in *Pelargonium zonale,* and in other plants where the inheritance is biparental though non-Mendelian, closely resembles that of albomaculata plants. The facts are as follows: Zonale plants are periclinal chimeras consisting of two somatically distinct layers of tissue; there may be a green core surrounded by an envelope of white, or the reverse. The form most studied is that with white-margined leaves in which the epidermal

and subepidermal layers are colorless. Sporogenous tissue in this form arises from the colorless tissue. When this type of plant is selfed or intercrossed, only white progeny is obtained. Reciprocal crosses with green are essentially alike, yielding variegated, white, and green seedlings in varying ratios. Baur found that variegation was confined to the seedling stage; the mature leaves were green or white, sectorial or periclinal chimeras. Green $F_1$ plants as well as green shoots on variegated plants gave perfectly normal offspring when selfed or crossed with green. The explanation originally advanced by Baur (2) for the results of crossing zonale and green was that two kinds of plastid were involved, plastids from the sperm cell being included in the egg cytoplasm. The fertilized egg cell thus possessed white plastids from the maternal parent and green plastids from the male, or the reverse. The two plastid types became separated by chance segregation during embryogenesis and the young sporophyte was at first variegated. Later, cell layers containing either white or green plastids were formed, thus giving rise to the chimeras found in mature leaves. Histological studies of the growing points of Pelargonium were in accord with the Baur hypothesis (see 26). Correns (7) stated that his hypothesis of an unstable cytoplasm did not apply to the type of variegation in Pelargonium and accepted the Baur hypothesis of mixed-plastid segregation. Here, then, we find a type of variegation which seems to depend upon the existence of distinct plastid types and which accordingly supports the concepts of plastid mutation and autonomy.

### Plastid Systems in Oenothera Species

The most convincing evidence that the plastids constitute an autonomous genetic system comes from the careful and detailed studies of Renner (25, 26) on Oenothera hybrids. Species of this genus not only differ in their genotypes but also have different plastid systems, which are adapted to specific kinds of nuclei and develop poorly with others; i.e., the plastid phenotype is a result of the joint interaction of plastogenes and nucleus. In the course of evolution the different plastid systems became differentiated by mutation as did the gene complexes. An example from Renner's extensive investigations is as follows: Genomes from *Oenothera muricata* and *Oe. Hookeri* were combined to give ᴴHookeri × curvans hybrids. (Hookeri has only one type of gamete while muricata has both the rigens and curvans complexes; we will follow only the curvans complex from muricata.) From the cross with Hookeri as the female parent, the hybrids ᴴHookeri × curvans were yellow and died. It was concluded that the plastids from Hookeri were not adapted to the hybrid nucleus. Hybrids that were of the same genic constitution but received their plastids from muricata were green, indicating that muricata plastids were adapted to the

hybrid nucleus. Renner found, however, that these green hybrids developed yellow flecks. He assumed that the sperm cell brought some plastids from the paternal parent into the embryo sac and that these plastids were included as a mixture with the more numerous maternally derived plastids in the cytoplasm of the fertilized egg. He argued that somatic segregation of the two plastid types resulted in the yellow areas of the green hybrids containing only plastids from Hookeri. Occasionally flowers arose from tissue having these Hookeri plastids, so that breeding tests were possible. When selfed, these yellow shoots gave two kinds of progeny: (1) ᴴHookeri × curvans, which were yellow as expected owing to the lack of adaptation of Hookeri plastids to the hybrid nucleus; and (2) ᴴHookeri × ᴴHookeri, which were green because the Hookeri plastids were asociated with a Hookeri nucleus. The Hookeri plastids from the yellow areas developed in the characteristic manner of Hookeri plastids when later combined with a different kind of nucleus. Mutation had not occurred, but their development had been impaired. Furthermore, the colorless plastids of ᴴHookeri × curvans plants behaved in other controlled crosses in the same manner as true Hookeri plastids.

Renner found that plastids from one species could be combined with foreign nuclei for 14 generations and then recovered with no perceptible change in their specific properties.

A weak link in the chain of Renner's argument is that it has not been possible to differentiate morphologically between the plastids of different species. The proof that the yellow flecks on green hybrids have plastids from the pollen parent rests upon extensive and consistent breeding data. It is difficult to distinguish, however, for any case of variegation, between a cytoplasmic and a plastid effect, since plastids and cytoplasm are concomitantly acquired. Renner rejects the theory of cytoplasmic effect, on the grounds that it would be difficult for the maternal and paternal components of a relatively fluid mass of cytoplasm to become separated, while the separation of the discrete plastids would be mechanically much more easily achieved. On the other hand, the chance segregation of two kinds of plasmagene should occur with the same readiness as that of plastids. But these postulated plasmagenes are only hypothetical entities, and Renner's conclusions on the existence of plastids as independent cytoplasmic units should be at least provisionally accepted.

### Gene-Induced Plastid Mutations

There are hundreds of examples of gene-controlled plastid characters. In maize, for example, there are more than a hundred genes that produce a wide range of mutant phenotypes such as whites, yellows, stripes, etc. In many of these cases it is likely that the gene effect has been merely the pro-

duction of a physiological condition unfavorable for normal plastid development; it is also possible that mutations of the plastogenes have been induced. That mutation of the plastogene can occur through genic action has been reported by Rhoades (*27*) for maize, by Sô (*28*) and Imai (*17*) for barley, and by Pal (*23*) for rice. A description of the maize case will be the most instructive. Iojap (*ij*) is a recessive gene located in chromosome 7. Homozygous *ij* plants are essentially green-and-white striped, although some yellow and pale-yellow stripes appear. When *ij* plants are used as the female parents in crosses with green, the $F_1$ populations from some crosses consist entirely of green individuals, while others consist of green, white, and striped plants in widely varying ratios. Rarely, an all-white progeny is found. The reciprocal cross yields only green offspring. It seems clear that green plants come from egg cells with normal plastids, and that white seedlings arise from zygotes having plastids incapable of normal development. The striped seedlings presumably come from egg cells having both normal and abnormal plastids in the cytoplasm.

The appearance of white and striped seedlings in the $F_1$ progenies where *ij* was the maternal parent can be interpreted as indicating that the *ij* gene is able to induce an irreversible change in the plastids. They are unable to develop normally in cells heterozygous for the dominant allele of *ij*. The occurrence of striped $F_1$ plants made it possible to carry the analysis one step further and to show that these plastids were not able to develop even when the *ij* allele was replaced by two normal alleles. This was accomplished by crossing the striped $F_1$ plants with green pollen. In a number of cases the ensuing progenies consisted wholly of white seedlings (see Table 1 for a sample of these data). These all-white populations, which arose from a white sector on the $F_1$ plant, should consist of equal numbers of $+ +$ and $+ ij$ plants. That $+ +$ individuals were represented in the population was shown by marking the *ij* chromosome used in the parental cross with the recessive glossy-1 (*gl*) gene, which is about 12 units from *ij*. Pollen of $+^{gl} +^{ij}$ was used, so that the striped $F_1$ plants were of $gl\ ij/+^{gl} +^{ij}$ constitution. The pollen used on the $F_1$ silks was $gl\ +^{ij}$, so that $gl\ +^{gl}$ and $gl\ gl$ individuals were produced in equal numbers. The non-glossy seedlings, barring crossing over between $gl$ and $ij$, were homozygous for the normal allele of *ij*. Since the two types of plant occurred in equal numbers among wholly white progenies, it was clear that the mutated plastids were unable to revert to normal in plants with two normal alleles.

A cytological study of the striped areas of *ij* plants gave some information about the action of the *ij* gene. The plastids in pure-green stripes were apparently normal in size and pigment content, while the colorless cells had minute plastids devoid

of both chlorophyll and carotenoid pigments. It was invariably observed that the normal-sized plastids in those green cells adjacent to white tissue were distinctly paler in color than were the plastids in cells further removed from the white cells. (This effect of colorless cells upon green ones observed here is not found in certain other cases of green-white mosaic plants.)

Chimeras where a layer of mesophyll cells with normal-sized plastids lies over a layer of colorless

TABLE 1. EXAMPLES OF BREEDING BEHAVIOR OF STRIPED $F_1$ *Ij ij* ♀♀ × *Ij Ij* ♂♂

| Family | Proportion of White Tissue in Maternal Parent | Classes of Offspring | | |
|--------|--------|--------|--------|--------|
| | | Green | Striped | White |
| 9682 | ca. 50% | 82 | 0 | 0 |
| 9683 | ca. 50% | 201 | 0 | 0 |
| 9686 | ca. 50% | 326 | 0 | 1 |
| 9687 | ca. 50% | 0 | 0 | 160 |
| 9688 | ca. 50% | 200 | 2 | 140 |
| 9689 | ca. 50% | 211 | 0 | 0 |
| 9692 | ca. 50% | 400 | 0 | 0 |
| 9694 | ca. 50% | 0 | 0 | 96 |
| 9697 | ca. 50% | 0 | 0 | 107 |
| 9698 | ca. 50% | 0 | 0 | 52 |
| 9699 | ca. 50% | 117 | 0 | 0 |

cells result in the appearance of yellow stripes. The color of these plastids, however, is distinctly paler than that of plastids in cells not adjacent to the colorless layer. This suggests that there is some substance in the colorless cells, responsible for the abnormal plastids found there, which is able to diffuse across cell membranes and impair chlorophyll development in normal cells. Indications of plastid disintegration were sometimes observed in plastids of the pale-colored cells. The action of the *ij* gene would thus appear to be upon the cytoplasm rather than directly upon the plastids. Possibly the degree to which the cytoplasm of a cell has been modified by the *ij* gene determines the fate of its plastids. They may not undergo mutation but only fail to develop normally when the cytoplasm has been modified relatively little, while a greater change in the cytoplasm results in irreversible plastid mutation. Judging from the proportion of white tissue present in iojap plants, it seems likely that not all colorless cells contain mutated plastids but that some possess plastids whose growth has been impaired, because the frequency with which white seedlings are found among the offspring is much lower than that expected if mutated plastids were present in all white areas.

If the *ij* gene modifies the cytoplasm and thus indirectly affects the plastids, how can we differentiate between irreversible plastid mutation and a permanently changed cytoplasmic condition in which normal plastid growth is impossible? This is a critical point and one on which decisive evidence

is difficult to obtain. It is essentially the same problem met with in considering "status albomaculatus," and there the evidence did not favor the occurrence of plastid mutation.

Whether or not the *ij* gene induces plastid mutations or the production of an abnormal plasmagene controlling plastid development, it is clear that self-duplicating bodies with non-Mendelian heredity result from the action of the *ij* gene. The occurrence of striped $F_1$ plants indicates that the cytoplasm of the egg cells from which they arose possessed either two kinds of plastids or two kinds of plasmagenes, and that somatic segregation does occur. That all the plastids in a single cell are not simultaneously transmutated is also shown by the finding of striped individuals. It is possible that too much emphasis has been placed on the cytological observations of plastids in iojap plants, and that the simplest explanation—that of plastid mutation—is the correct one.

There is no reasonable doubt that gene-induced cytoplasmic mutations occur, that the cytoplasm or, more likely, some particulate part thereof can be permanently modified by specific genes. Two significant studies, which have some relation to the iojap story, have recently been published. Spiegelman, Lindegren, and Lindegren (30) found an adaptive enzyme in yeast that was gene-initiated but self-duplicating in the presence of substrate even though the activating gene was absent. Sonneborn (29) found that the K gene in *Paramecium aurelia* could form a cytoplasmic substance, kappa, when kappa was already present in the cytoplasm, but could not initiate the formation of kappa.

Imai (17) described a case of variegation in barley that apparently involved both exo- and automutation of plastids. (Imai uses the term exomutation for gene-induced plastid mutation and automutation for spontaneous plastid mutation.) In a strain homozygous for a recessive gene causing recurrent exomutations of green plastids to white and producing a green-white striped phenotype (28, 15, 16), Imai found a single plant with green, yellow, and white stripes—i.e., it was tricolored. The green areas had white stripes caused by exomutation of green to white, and the yellow areas had white stripes caused by exomutation of yellow plastids to white. White stripes were therefore found in both green and yellow tissue. The yellow areas also exhibited green stripes due, according to Imai, to automutation of yellow to green, and within these green-striped regions smaller white stripes were found owing to exomutation of green to white. The yellow plastids in the original tricolored plant presumably arose through automutation of green to yellow. Briefly, both green and yellow plastids were exomutable to white, and yellow plastids were automutable to green. The breeding behavior of the tricolored plants indicated that the inheritance of both yellow and white plastids, the first arising by exo-

mutation and the second by automutation, was non-Mendelian and tranmitted solely through the maternal cytoplasm. Imai's interpretation is consistent with the data, but can hardly be said to be rigorously established.

Yasui (33) reported on the inheritance in Hosta of a tricolored form that had green, white, and yellow tissue. The inheritance of the three plastid types was non-Mendelian. The simplest explanation is that this strain contained three distinct and separable kinds of plastid; but, as de Haan (9) has pointed out, other explanations are not excluded. A more intensive genetical study of this form in conjunction with a careful cytological study is necessary before Yasui's conclusions can be accepted.

Imai (18) has taken a curious position with respect to those gene-controlled variegations that produce no white offspring. He assumes that the white regions of the variegated plants arise from recurrent mutations of green plastids to white and that the failure of the white plastids to be transmitted cytoplasmically is due to their spontaneous mutation to green during gametogenesis. A more likely explanation is that mutation does not occur at all but that the physiological conditions in certain tissues are unfavorable to normal plastid development.

In connection with the problem of plastid mutation, mention should be made of Woods and du Buy's hypothesis (32) that through extreme mutations plastids may be transformed into virus-like particles consisting only of nucleoprotein and possessing invasive powers. Such, indeed, may be the case; but their published data do not warrant so far-reaching a conclusion. They have not shown that the variegations which were graft-transmissible were not themselves due to the presence of a virus. Furthermore, the intermediate stages of chloroplast breakdown which they observed in the transition zones between green and colorless areas, and which they attribute to invasion by virus-like particles arising by plastid mutation, are paralleled by the conditions found in transition zones in iojap leaves.

### IRRADIATION-INDUCED MUTATIONS

The evidence is good but not conclusive that the plastid system of the cells constitutes an independent element, with the individual members (plastogenes) able to self-reproduce and possibly to mutate. Comparatively little study has been made, however, of the effects of irradiation upon these cytoplasmic elements. This neglect is due in part at least to the difficulty of ascertaining in any given case whether the induced modification is in the plastids or in some other component of the cytoplasm. Nevertheless, here would appear to be a fruitful field of endeavor, especially with certain of the lower plants which have a small number of plastids and a characteristic morphology. That success may be expected seems indicated by the interesting results already obtained

in a number of cases. Knudson (*20*, and unpublished) treated the spores of the fern *Polypodium aureum* with X-rays and found a variety of plastid changes. Some of these maintained their mutant phenotype through the sporophyte generation and were recovered unchanged in the following gametophytic generation. While Knudson's results are of interest, he has not yet reported on the critical experiments of fertilizing the archegonia of gametophytes containing mutant plastids with male gametes from normal gametophytes and determining the plastid type in the ensuing sporophyte and in the subsequent gametophytes.

A number of investigators (*22, 14, 4*) found modifications in the appearance of plastids or mitochondria following irradiation. Nothing was determined, however, about the constancy or heredity of these changes, and these observations have little relevancy to the problem of plastid mutations. Various chemical treatments of plastids, summarized by Küster (*21*), produced abnormalities; but the permanence of these modifications was not ascertained. Stadler (*31*) found three barley plants with variegated tillers in a population from X-rayed seed. The breeding behavior was that of a maternally inherited plastid variegation; evidently a cytoplasmic change had been induced, but whether it was the plastids or some component of the cytoplasm that was affected is unknown.

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# BIOLOGICAL PHASE MICROSCOPY

## OSCAR W. RICHARDS

Living organisms and tissues reveal little of their complex structure with the ordinary brightfield microscope. To see them, the condenser diaphragm of the microscope must be nearly closed so that only a narrow cone of light is used for illumination. As the illuminating cone is decreased, contrast improves until the diffraction patterns become so large that the finer details are obscured and the resolving power is lost. Small specimens may never be seen, because the resolving power may be lost before enough contrast is developed for visibility. Consequently, elaborate staining procedures have been devised for seeing the finer structure with the microscope. Vital staining is limited, and most of our knowledge has been restricted to post-mortem observation.

The structure of the uncolored, living organisms includes regions of different optical path owing to differences in size and refractive index. The *optical path* is defined as the distance the light passes through the region multiplied by its refractive index. Light passing through such a region is slowed or speeded as the optical path is increased or reduced, and the phase relations of the light waves are altered. Our eyes are insensitive to the phase differences, but do respond to intensity differences. A ray of light (Fig. 1A), on passing through a region of greater optical path (B), is slowed so that the crests and troughs are behind and out of phase with those of a corresponding ray (C), which does not go through the region of greater refractive index. The light ray (D) passing through an absorbing medium (E) is reduced in amplitude, but remains in phase with ray (C).

The *Phase Microscope* controls the light passing through it so that phase differences originating in the specimen are converted into visible amplitude, or intensity differences. *Phase Microscopy* is a method for increasing or decreasing the contrast in the image by utilizing absorption and optical path differences within the microscope. This provides another microscopical method, and—like brightfield, darkfield, polarization, optical staining, epi-illumination, fluorescence, and other methods—it has unique advantages and limitations, which will be discussed briefly.

## HISTORY

The interactions of light waves with each other have long interested physicists, and Bratuscheck (4) studied phase relations within the microscope. Rhineberg (18, 19) and Conrady photographed a grating using a phase difference microscope. Zernicke (24, 25) of Groningen, developed a vector analysis for phase relations and discussed its implications for microscopy. He persuaded the Zeiss-werke to develop phase-contrast equipment for the microscope, which was described by Köhler and Loos (14) and marketed in Europe about that time. Burch and Stock (6) successfully used a slit-type of Zernicke plate for biological microscopy in England.

The development of the Spencer phase microscope was retarded by the war; it was publicly demonstrated in 1944 (1). Not finding the vector analysis satisfying, Bennett, Osterberg, and Jupnik developed a general theory and terminology for four types of improved diffraction plates, which extended greatly the usefulness of the phase microscope. For a more detailed history and discussion of the mathematical analysis the reader should consult Bennett *et al.* (2).

Köhler and Loos (14) found the phase microscope useful for the observation of urine sediments, fresh blood, trypanosomes, epithelial cells, and sections of kidney and tooth. Loos (15) extended his observations and published phase photomicrographs of staphylococcus, diphtheria bacteria, connective tissue, fibroblasts, tumor cells, yeast, and fungi. Michel (16) recommended the phase microscope for the study of germ cells, and reported making a motion picture of spermatogenesis of the grasshopper and observing unstained salivary-gland chromosomes. The superiority of phase over brightfield microscopy was demonstrated by Bosshard (3) for lactic-acid bacteria in beer, yeast, sarcina, trypanosomes, fungi, living Chironomous chromosomes, and brain tissue. Brice *et al.* (5) reported seeing the Golgi apparatus in smears of Lumbricus seminal vesicles. These observations were made with Zeiss equipment, which consists of a condenser with interchangeable diaphragms and 16-, 4-, and 1.8-mm. objectives, each with a single built-in diffraction plate.

Burch and Stock (6) suggested using epithelial cells for demonstrations, and stated that their slit-type diffraction plate showed these and spirochaetes well. This kind of diffraction plate is not symmetrical, and has been used less than the circular type. While a careful attempt has been made to find all the literature in this field, a complete survey of the European literature is not possible now and some contributions may have been missed.

The Spencer Phase Microscope was reported successful for seeing unstained living bacteria, mold, protozoa, fibers, blood, industrial materials, and epithelial cells (21). The original bright-phase photomicrograph of frog epithelial tissue showed regions that some observers considered to be the Golgi apparatus. The destruction of Rhizobium by its bac-

teriophage was seen by Hofer and Richards (*11*). Harrison *et al.* (*10*) made in color a phase cine-photomicrograph of the immune reaction of *Paramecium bursaria* which has been described by Harrison

and Fowler (*9*). Farr (*8*) reported the advantage of phase microscopy for the investigation of the structure of the cotton fiber.

## THE PHASE MICROSCOPE

The microscope has an annular diaphragm (Fig. 1K), at the front focal plane of the condenser, to limit the light to a hollow cone of proper size, and a diffraction plate (J′) at the back focal plane of the objective. Otherwise the microscope is unchanged. In the 16-mm. objective shown, the diffraction plate goes between the lens systems. In the 4-mm. objective the focal plane is at the outer surface of the back lens and the diffraction plate is held there by a suitable cell mount. The focal plane in the 1.8-mm. objective is within the lens system; the diffraction material is placed on the proper lens surface and each objective is limited to only one plate. The diffraction plates for the 4- and 16-mm. objectives are removable, and different ones may be used successively in the respective objectives. The annular diaphragms for the condenser are also interchangeable, and one is required to match the numerical aperture of each objective used.

In use, the image of the condenser iris is centered to the ring of the diffraction plate; this is easily done while observing these with an auxiliary telescope in place of the microscope eyepiece. The diaphragm is inserted into the condenser and likewise centered. Separate centering screws are provided on the condenser mount for each. The telescope is removed, the eyepiece replaced, and the instrument is ready for use.

Light from any point (L), likewise from every point in the opening, passes from the condenser to the objective in parallel bundles, which come to a focus and must pass through the conjugate ring of the diffraction plate. The light may be partially absorbed or retarded in accordance with the material placed on the ring of the diffraction plate. When a specimen (N) is present, it diffracts light, shown by the dotted bundle in Fig. 1, which passes mainly through the rest of the diffraction plate. The specimen light is imaged in the ocular at (Q), where the destructive interference takes place and the phase differences are changed into visible intensity differences. When light rays are in phase they combine to give a brighter image. If they are exactly out of phase they destroy one another and the absence of light is seen as black. Intermediate relations give a series of grays. This result concerns physical rather than geometrical optics. A mathematical formulation of the phase relations in the image may be found in Bennett *et al.* (*2*) and will not be repeated here. It is shown therein that the resolving power is usually not decreased and the image formed in the phase microscope may be a truer representation of the specimen than that obtained by other methods.
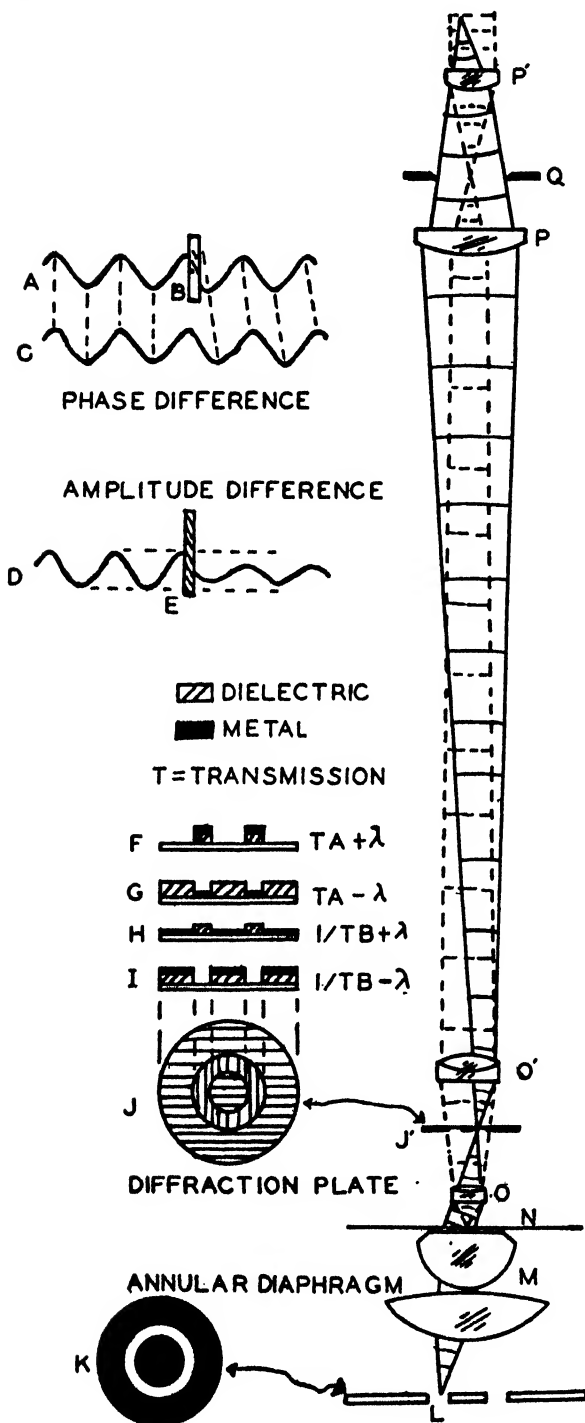


FIG. 1. A-E, phase relations and absorption in light waves. F-J, diffraction-plate types. L-P, diagram of the phase microscope. (*cf.* text.)

## DIFFRACTION PLATES

The diffraction plate is a piece of optical-grade glass having evaporated on it, in vacuo, a dielectric material to retard light, a metal to partially absorb light, or both. When the metal is placed only on the ring (Fig. 1FG), it is called an A-type diffraction plate; when placed elsewhere than on the ring (HI), a B-type. When the dielectric is placed on the ring, the optical path for the light not deviated by the specimen is increased (FH) and a plus sign is added to the letter showing the kind of diffraction plate. Placing the dielectric elsewhere than the ring (GI) increases the optical path for the light deviated by the specimen, and the minus sign is used. The ratio of the transmission of the undeviated light to that of the deviated light is indicated by a decimal fraction (T) placed before the letter (F-I), which is less than unity for A- and greater than unity for B-type plates. The amount that the light is retarded by the dielectric, when present, is indicated in fractional wave lengths of light of 546 m$\mu$ after the letter. For example, a $0.25A + 0\lambda$ plate absorbs 75% of the undeviated light and does not affect differentially its phase relations. A $4B - 0.25\lambda$ plate transmits 25% (1/T) of the deviated light, which is retarded by a quarter wave length with respect to the undeviated light. A $1A - 0.1\lambda$ transmits all of the undeviated light and retards by a tenth wave length the light deviated by the specimen.

The harshness of the contrast is influenced by the width of the ring of the condenser diaphragm (Fig. 1K) and its conjugate, the width of the ring on the diffraction plate. This offers another means for controlling the contrast of the image in phase microscopy. The outside diameter of the ring, however, must be large enough not to reduce the numerical aperture of the objective. Otherwise, the resolving power of the objective will be impaired.

## PHASE MICROSCOPY

While a very large number of combinations with from 100% to a few per cent transmission, and retardations up to a wave length are possible, both theory and practical observation have shown that certain of these are more useful than others. The use of only a single diffraction plate, we believe, unnecessarily limits phase microscopy.

The A+ and B+ diffraction plates make regions of greater optical path brighter than those of lesser path; and conversely, the A— and B— plates make the regions of greater path darker. The former result will be called bright and the latter dark contrast. In general, bright contrast is preferable for counting and dark contrast for measuring. Some observers prefer dark contrast because the image more nearly resembles that seen in stained preparations. Some experience may be required with bright contrast, as this kind of image is less familiar to the microscopist. With bright contrast the smallness of

a particle that may be noted is limited only by the amount of light available. Thus, with bright contrast particles below the limit of resolution may be discovered, although not resolved as to size and shape.

When regions of different optical path are present certain diffraction plates will make some of them darker than others, which gives the optical equivalent of *differential staining*. Such regions may be reversed by the opposite type of diffraction plate and observed in either bright or dark contrast. When the optical-path differences in the specimen are large, darkfield microscopy is indicated, but when the differences are small as with many biological preparations, the phase microscope is preferable. Opaque specimens and strongly stained preparations are seen better in transmitted light with the brightfield microscope. Lightly stained preparations, especially if supravitally stained, may be examined, and the stain and phase contrast added for increased visibility. With transparent colored specimens, it is possible to combine color contrast between the specimen and the illumination, with that from the phase differences to improve visibility. Combinations of phase and polarization microscopy are also useful and will be reported elsewhere (see also, *17*).

A research-type lamp with a condensing lens and iris diaphragm should be used for best results. The filament image is focused on the annular diaphragm in the microscope condenser by focusing the lamp condenser, and the lamp iris diaphragm is focused in the plane of the specimen by focusing the microscope condenser. With diffraction plates of the greater transmissions, illuminators giving less light may be used when the visibility is satisfactory. For other than research-type lamps, the light should be adjusted until both the specimen and the aperture of the objective are uniformly illuminated. For fine detail and high contrast, monochromatic light may be used to advantage. The sodium arc or the mercury arc (*e.g.*, AH-4) with proper filters may be used.

Thin specimens or sections are better than thicker ones, because overlying material may contribute optical path differences that obscure the detail to be examined. Most of the standard methods for preparation of specimens may be used in phase microscopy, except that dense staining or metal impregnations are contraindicated.

The optical-path differences between the specimen and the mounting medium should not be large. A mounting medium of nearly the same refractive index as the specimen is preferable with phase microscopy. Living organisms and tissues show better in a physiologically normal medium such as nutrient, saline, Ringer, and similar solutions. Semipermanent preparations of bacteria and spermotozoa have been made with glycerine jelly, ringed with Gold Size. Unstained protozoa and paraffin sections mounted in Clarite have shown good detail (Figs.

17, 18). Textile fibers of higher index revealed more detail when mounted in immersion oil of nearly the same refractive index than in glycerine, water, or paraffin oil.

Relative contrast and visibility may be increased or decreased by using different diffraction plates, by changing the refractive index of the specimen or its mounting medium, or by varying all three. The latter variations may be accomplished by using successively media of different refractive index, diluting the mounting medium with a solvent, concentrating the medium by adding solids or evaporating the solvent, or by changing the temperature of the preparation.

Sufficiently isotonic media with different indices of refraction can be devised for living organisms by use of nontoxic large molecules, which do not penetrate the organisms. Aqueous solutions change fairly rapidly in refractive index with change in temperature, even within the small range in which life is possible. This will aid the biologist both with visibility and analysis of the effects of temperature on living systems. Concentration gradients vary in path and may be visualized, and microschlieren methods offer further possibilities for the experimental biologist. When appropriate, swelling and macerating methods are useful, especially when their action is watched. Running the reagents under the cover glass, or flowing-cell methods, may be used.

## APPLICATIONS

Phase microscopy is fast becoming a very useful tool for the biologist. Cytology and histology based on living rather than dead material are developing rapidly. The effects of fixation, staining, and other chemical treatments may be assessed. The process of dying may be watched. Physiological processes may be studied and the effects of many materials, both normal and toxic, can be observed now that these transparent, uncolored, organisms and their internal structure may be seen clearly. In clinical diagnosis, time for staining is saved; and it is very likely that in many conditions identification will be more certain as the sensitive method of phase microscopy is developed.

Measurement of the dimensions of bacteria and other small microorganisms is now possible (*22*). Living bacteria cannot be seen clearly because of the uncertainty at the boundary due to the wide diffraction patterns formed from closing the condenser diaphragm to give some contrast (Figs. 12, 13). The difficulties have been discussed by Knaysi (*13*); and Dubin and Sharp (*7*) tabulated sizes of the outer, inner, and middle zone of the diffraction pattern of the wall. Heat fixing and staining are known to shrink bacteria; negative staining has proved difficult; and it is not known how much bacteria may be altered in form when prepared for and during examination with the electron microscope. With the phase microscope, sharp cell boundaries may be observed with rapidly growing young cultures in nutrient broth or other favorable medium (Figs. 15, 16). Photomicrographic records (*q.v.* below) of motile forms will probably give the most precise measurements. Unless the image is sharp there seems little point in measurement. Arguments about the size of bacteria and other small microscopic specimens will be ended with correct information. Likewise, measurements of size changes in growth and studies of osmotic exchange can now be made with sufficient accuracy to be meaningful.

The chief difficulty of phase microscopy is in determining which of the many kinds of diffraction plate gives best results with the variety of materials observed with the microscope. Some of our information has been published by Bennett *et al.* (*2*), and other will be summarized here. While the knowledge is admittedly incomplete, it does suggest possibilities and point the way to further investigation. No single diffraction plate has been found satisfactory for all biological materials; and there is every reason to believe that different specimens and, in some cases, parts of the same specimen, will require different diffraction plates to reveal all of the structure. For some special applications a single plate is satisfactory. Since no comparable microscopic equipment has been built, comparisons are not possible. Most of the observations reported abroad have been confirmed and greatly extended. In reporting the following observations, the diffraction plate found useful will be indicated in the terminology described above. The writer has been privileged to examine many materials brought to his laboratory for the evaluation of phase microscopy and wishes here to express his appreciation to the scientists for their interest and aid in phase microscopy.

The results of varying contrast are shown in Figs. 2-9, showing a living *Paramecium bursaria*. (The focus was maintained, as nearly as possible, on the oral groove shown best in Fig. 3.) Without phase much of the detail was lost, as it was necessary to close the condenser from N. A. 0.66 to 0.4 to obtain contrast. The commensual algal cells, Fig. 4, show well with a $2.6B - 0.25\lambda$ diffraction plate and but poorly when the transmission is increased to $1B - 0.25\lambda$ (Fig. 6). The $0.25A + 0\lambda$ plate shows promise (Fig. 8), but much less detail than the $0.25A + 0.25\lambda$ (Fig. 3), which gives the greatest detail within the animal. Decreasing the transmission to $0.07A + 0.25\lambda$ increases the reversal to bright contrast and darkens the macronucleus (Fig. 5). Notice the difference between Figs. 8 and 9. Fig. 8 was made with a diffraction plate having three times more transmission than that of Fig. 9, yet both depend on absorption differences alone as no retarding material was present in the plate. Cilia show better with $0.2 \pm 0.25\lambda$, but did not photograph as well with the high-speed lamp as the heavy diffraction patterns of the cilia without phase. (Longer exposures would have corrected this, but would have given less detail

within the animal.) Which diffraction plate gives the best contrast in the image, depends on what structure is to be examined. To comprehend the whole animal requires more than one type and kind of contrast. For this animal the seven shown are probably the best, although other detail can be intensified or suppressed by still other diffraction plates.

Bacteria show well in ordinary media (Figs. 15 and 16) with 15 to 25% A ± 1/4λ diffraction plates. With the same retardation, but one-third the transmission, the structure within the cells is more clearly seen. The 2.3B — 0.33λ is useful also. The destruction of Rhizobium by its bacteriophage was seen by Hofer and Richards (11) using a 0.17A + 0.1λ diffraction plate. For bacteria the magnification of the oil-immersion objective is necessary to see cellular detail, but the larger species may be seen well enough to count with the 4-mm. objective. Unstained rickettsiae in smears were seen better with 1B — 0.25λ, although the 0.2A + 0.25λ was good. Figs. 17 and 18 are of an unstained paraffin section of liver from a rat infected with Borella, which we believe is shown at the points indicated by the arrows.

Yeasts are revealed by 2B — 0.33λ, 0.4A + 0.33λ, and 0.15A + 0.1λ diffraction plates; and Lindegren has discussed the usefulness of phase microscopy in another paper of this Symposium. Fungi require different diffraction plates for optimal observation, as the optical-path changes are proportionally greater in the larger than the smaller species. The large granules in the cytoplasm and the septa of *Aspergillus niger* were seen better with a 0.35A + 0.25λ diffraction plate and the finer granules with a 1A + 0.25λ plate. The conidia and spore arrangement was shown better with a 1.33B — 0.38λ plate. Good bright contrast for a Penicillium occurred with a 0.25A + 0.25 plate and dark contrast with a 1.3B — 0.38λ diffraction plate.

Algae and Diatoms vary in size and optical path. Some of the more useful diffraction plates are 0.25A + 0λ, 0.15 to 0.5A + 0.25λ, 0.15A — 0.25λ, 2B — 0.1λ, 2.6B — 0.25λ, and 2.3B — 0.33λ.

Cyclosis in Elodea and in the epithelium from the inner scales of the onion bulb (Fig. 10) show very well under the phase microscope. The protoplasm of the latter reveals mitochondria and the various kinds of plastids reported by Sorokin (23), without recourse to vital staining, when a 0.2A + 0.25λ plate is used for bright and a 0.2A — 0.25λ plate for dark contrast.

Bright contrast is preferable with protozoa for making counts, and was found useful by Harrison and Fowler (9) to show the exudation of mucus-like material that entangles the cilia of sensitized *P. bursaria* placed in antiserum. Owing again to the wide varieties of size of protozoa and the changes in image contrast with various diffraction plates (Figs. 2-9), no general recommendations can be made. For parasitic protozoa (*E. histolytica, Balantidium coli,*

*Endolimax nana*) the 0.2A + 0.25λ and 2.6B — 0.4λ give increased contrast, which facilitates finding and identifying the parasites. *Taenia saginata* may also be seen with the latter plate. Malaria sporozoites can be seen in unstained blood smears (Fig. 11) with a 0.13A + 0.25λ plate.

Spermatids and spermatozoa are revealed in dark contrast by 0.15A — 0.25λ plate, in bright contrast by a 0.2A — 0.25λ plate (Figs. 14, 20, and 21) without the diffraction blurring seen with brightfield (Fig. 19). The photomicrographs were made of unstained, living sperm with exposures of about 1/35,000 second.

Nucleated embryonic erythrocytes and leucocytes are seen with a 0.2A + 0.25λ plate, and the latter show well in dark contrast with a 0.15A — 0.25λ diffraction plate. Supravitally stained white cells show far more with a 1B — 0.25λ diffraction plate than with brightfield microscope, both in detail and color differentiation of the cellular constituents. The latter plate is useful also with unstained connective tissue. Myelin sheaths show with 0.15A — and 0.2A + 0.25λ diffraction plates. These same plates are good for epithelial cells of animals, and apparently show the Golgi apparatus of some unstained living animal cells. Fig. 22 shows a bright-contrast, low-power photograph of a mite, Bryobia, mounted in Clarite.

Salivary-gland chromosomes are revealed without staining (Figs. 23 and 24) using a 0.15A + or 0.12A — 0.25λ diffraction plate. The dark contrast of the latter is preferred by some observers. Chromosomes in Tradescantia stamen hairs, however, are seen better with a 2.3B — 0.33λ diffraction plate. Division figures can be seen in preparations of living cornea even though they are covered by layers of epithelial cells.

Textile fibers may be studied to advantage with the phase microscope, especially the spirals of young cotton fibers. Swollen preparations are useful. Surface casts of hairs in cellulosic material should have greater visibility with the phase microscope. Emulsions such as homogenized milk and mayonnaise reveal particles about a quarter smaller when examined with the phase microscope. Crystals can be seen clearly when so close in refractive index to the material surrounding them as to be invisible with the brightfield microscope. Transparent surfaces may be examined with the phase microscope.

The above brief summary of a small part of our experience with the Spencer Phase Microscope is given to suggest some of the more promising applications in biological science and medicine. So far, certain specimens have been found that show better with one or another of the four types of diffraction plate (Fig. 1F-I), although little experience has been obtained with the B+ type. Some 70 different combinations have been made and investigated. The quarter-wave retardations have proved most useful, as may be expected from the theory,
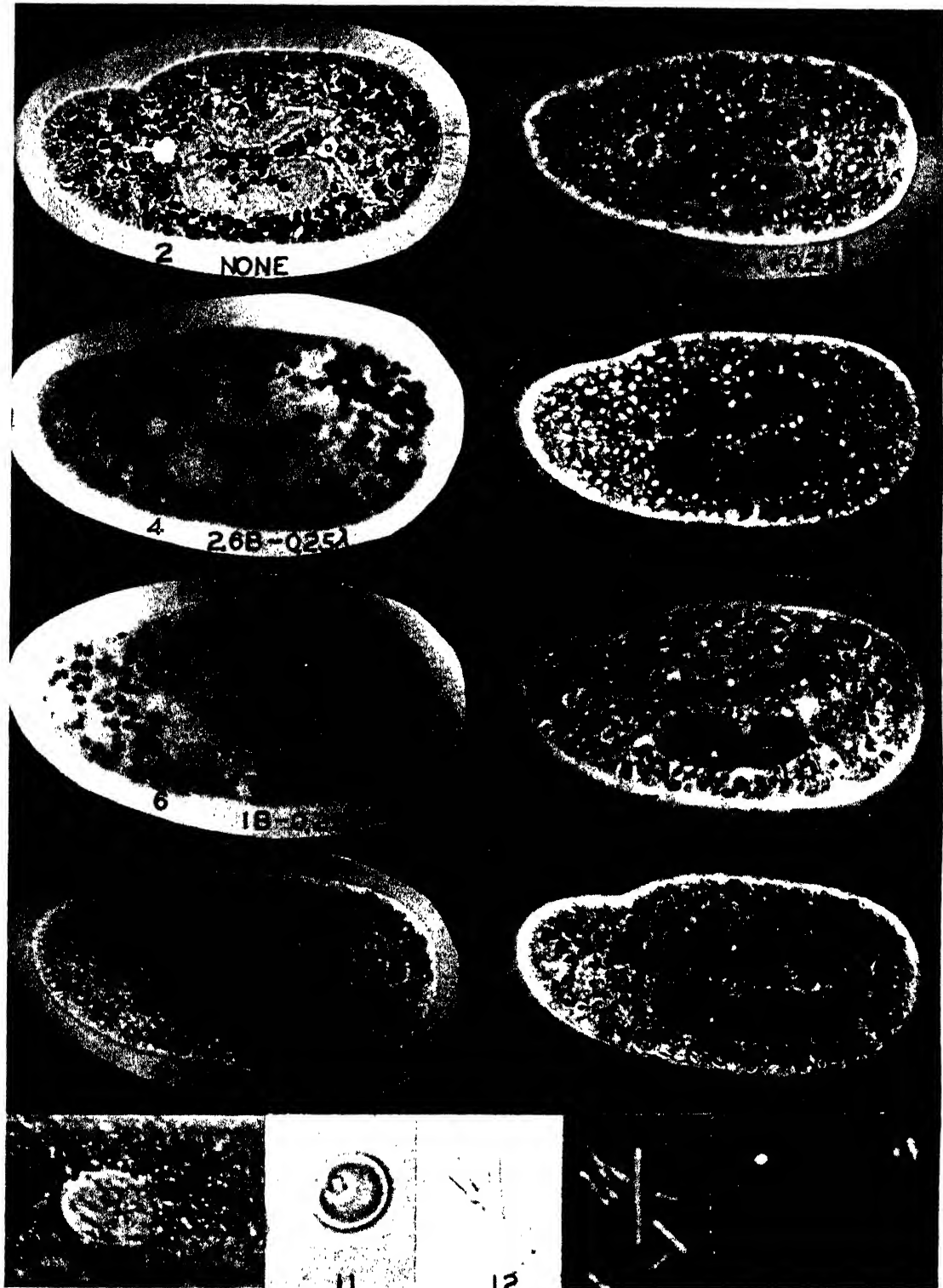
FIGS. 2-9. Photomicrographs of an unstained, living *Paramecium bursaria*, to show the appearance with the indicated diffraction plates. 550 ×.

FIG. 10. Living, unstained epithelial cell of onion, showing plastids and a mitochondrium. Bright contrast. 500 ×.

FIG. 11. Sporozoite of *Plasmodium vivax* in red blood cell; dark-contrast phase micrograph. 1300 ×.

FIG. 12. Living, unstained *B. megatherium* in bright field. Note obscured boundaries from diffraction. 1300 ×.

FIG. 13. Living, unstained *B. megatherium* in bright contrast with sharp boundaries. 1300 ×.

FIG. 14. Living, unstained sperm in bright contrast. 1250 ×.
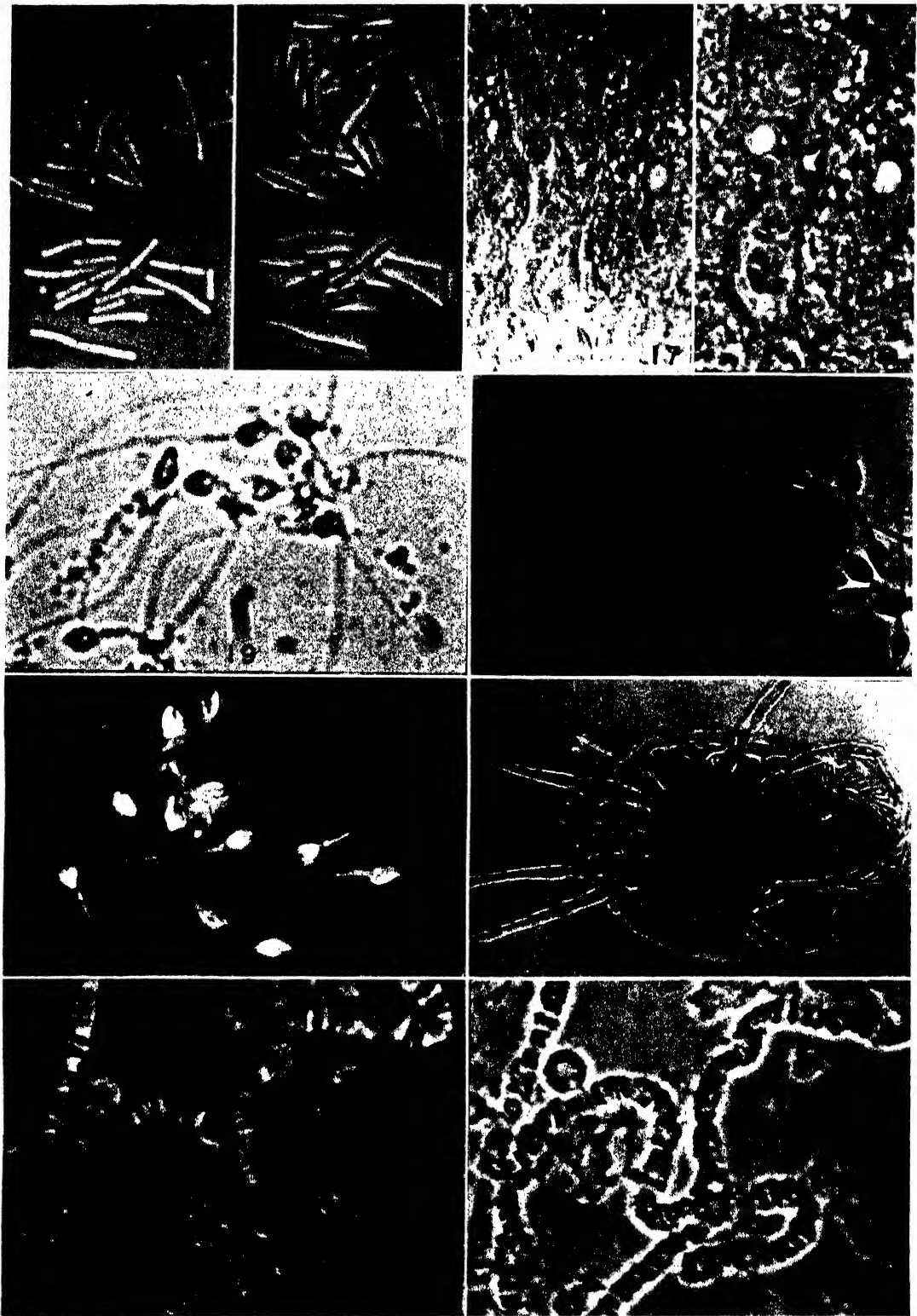
FIGS. 15, 16. Living, unstained *B. cereus* in bright and dark contrast. Note sharp cell boundaries. 1300 ×.
FIGS. 17, 18. Section of rat liver with brightfield and bright-contrast phase microscope. Arrows indicate Borellia. 1100 ×.
FIG. 19. Living unstained spermatozoa. Brightfield microscope. Note cell details obscured by diffraction. 1250 ×.
FIGS. 20, 21. Living, unstained spermatozoa. Dark- and bright-contrast phase micrographs showing sharper details. 1250 ×.
FIG. 22. Mite, Bryobia; Clarite mount. 100 ×.
FIGS. 23, 24. Unstained Drosophila salivary-gland chromosomes. Bright- and dark-phase micrographs. 1300×.

which predicts that a change of a half wave will give the reverse contrast in the image formed with the microscope.

Absorption without retardation is to be preferred with some crystals, diatoms, tissues, and unicellular forms. Low transmissions give better detail in the A series of plates. Retardations of less than and more than a quarter wave are better for some specimens. In the B plates, retardation without absorption is especially good with supravitally stained preparations. Dark contrast with A and B plates is different, and the choice depends both on the nature of the examination and on the material. For the present it seems best to make the phase equipment available on a prescription service basis, rather than to attempt premature generalizations. As experience and theory are developed, it will be possible to predict in advance from known path differences the plate to use, and to determine the refractive index of biological structures when their size is known.

### Photomicrography

Photographs may be taken with the phase microscope, using the same equipment, materials, and technic as with other kinds of microscopy. With diffraction plates of 1A or 1B the exposures are about the same as at full aperture without the diffraction plate. With 0.25A or 4B plates the exposures are about the same as for brightfield with the microscope condenser iris closed enough to give adequate contrast. With diffraction plates of lesser transmission the exposures will be proportionally longer. The transmissions of the diffraction plates are based on the area ratios of the ring and the rest of the diffraction plate; and, while descriptive of the plate, they do not indicate the amount of light passing to the camera and should not be used as guides to photographic exposure. Stereophotomicrographs are especially useful in recording the differential detail revealed by the phase microscope. Details will be published elsewhere.

With emulsions and smaller particles in living organisms, the Brownian movement blurs the pictures taken with ordinary tungsten microscope illuminators. Cell movements—as cilia, flagella cyclosis, etc.—are often too rapid to permit exposure with usual equipment. To make photomicrographs a very intense source is needed for rapid exposures. Sources are being developed. Movement was stopped in Figs. 12-16 and 19-21, taken at about 1/35,000 second using an experimental flash tube. (To Mr. Frank Carlson, General Electric Co., Nela Park, Cleveland, who made these experiments possible, go my hearty thanks.)

Now that the finer structure of living organisms can be seen and photographed, important biological information may be obtained from the analysis of motion-picture records made with the phase microscope. Cell division, sol-gel reversals, cyclosis, and other functional changes offer direct challenge.

The increased visibility at full aperture and with full resolution should be useful for microdissection studies, which should be recorded as motion pictures for study and demonstration. The phase microscope gives the biologist a new tool for direct investigation of living material in normal environments, which promises answers to many questions perplexing at present. The possible rewards more than justify solving the problems that confront the pioneer in this newly opened field.

*Note added in proof:* Since this was presented, E. Ganz (Vjschr. naturf. Ges. Zürich 89: 268-280, 1944) has published a general description of the Zeiss equipment and its use, and A. v. Albertini (Acta Anat. 1: 463-468, 1946; Schweiz. Z. Path. Bakt. 8: 289-310, 1945; Praxis, Schweiz. Rund. Med., rep. 19 pp., 1946) has stressed the use of phase microscopy for the examination of fresh preparations of epithelial cells, exudates, muscle and connective tissue, and smear preparations of tumors. Surface structure of cells was examined and reported. A. H. Bennett has published a brief history (Sci. Mon. 63: 191-193, 1946) and application to examination of crystals. The use of replica technics in phase microscopy was reported by O. W. Richards (Electron Microsc. Soc. Amer. 12-6-46: 8-9).

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# BACK-MUTATION AND ADAPTATION OF NUTRITIONAL MUTANTS

FRANCIS J. RYAN[1]

The change from a heterotrophic to an autotrophic growth-factor habit can be brought about experimentally in both bacteria and fungi. In an anthropomorphic sense this advantageous adjustment to a deficiency in the environment may be called an adaptation. In many cases such nutritional adaptations are inherited. The cause of these nutritional changes has been obscure, mainly because of the lack of adequate methods for discriminating between two major hypotheses, both of which were consistent with the facts. The first of these assumed the occurrence of "spontaneous" mutants, which
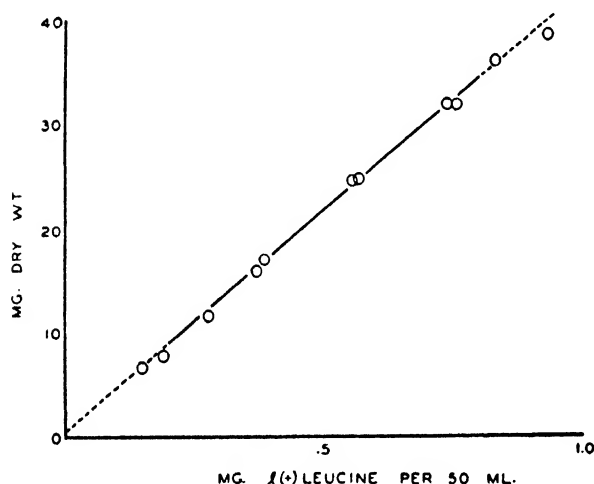


FIG. 1. The relation of leucine to the dry weight of mycelium produced by leucineless Neurospora in 50 ml. of liquid medium after 8½ days at 30° C. (Ryan and Brand, 1944.)

could utilize the simpler medium and so give growth. The second explanation was that adaptation is a direct response of the organism to the chemical stimulus of changed nutrient conditions. In the words of Knight (12), "new enzymes are produced as a direct reaction to the chemical stimulus of the new nutrients, in the absence of the normal nutrients."

Our interest in this problem was aroused in the course of an attempt to use mutants of Neurospora for the assay of amino acids. This study mainly concerned a leucineless mutant, 33757, which was

obtained at Stanford after treatment with ultraviolet radiation. Its inability to synthesize leucine is caused by a difference from the wild-type mold in a single gene (20). In order to grow, the mutant needs, in addition to the wild-type requirements of sugar, salts, and biotin, the amino acid, leucine. The extent to which growth takes place in liquid medium depends upon the amount of leucine present. Fig. 1 shows the linear relationship that exists at 30° C. between the final amount of mycelium produced by the leucineless mutant and the amount of leucine that supported the growth. The dry weight of the leucineless mold depends upon the leucine content of any protein hydrolysate on which it is grown. In this fashion analyses of pure proteins were obtained which compared very favorably with values secured on identical preparations by the most reliable chemical methods (24). The Neurospora method for the determination of leucine has been used in the first complete analysis of a crystalline protein (6) and for other purposes (5).

Although it does not affect the reliability of the results, there is one disconcerting aspect of the leucine method. This is the occurrence of adaptations. Some cultures did not show the usual relationship to leucine but grew like "wild-type," independent of leucine. Their weights did not fall on the curve shown in Fig. 1 but were higher to varying extents than the weights of similar cultures (Table 1). Without an understanding of their na-

TABLE 1. COMPLETE AND PARTIAL ADAPTATIONS OF LEUCINELESS NEUROSPORA AT 30° C.

| Flask Number | Mg. Dry Mycelium on Different Amounts of Leucine | | |
| --- | --- | --- | --- |
| | 0.25 mg. | 0.50 mg. | 1.00 mg. |
| 1 | 9.8* | 17.5 | 32.7 |
| 2 | 7.3 | 17.4 | 32.1 |
| 3 | 7.3 | 44.8* | 32.9 |
| 4 | 7.3 | 19.0* | 31.2 |
| 5 | 16.2* | 17.8 | 33.2 |
| 6 | 20.8* | 17.2 | 33.5 |
| 7 | 7.3 | 17.5 | 34.6 |
| 8 | 7.1 | 18.0 | 32.6 |
| 9 | 8.0 | 20.1* | 35.4 |
| 10 | 50.3* | 17.3 | 35.1 |
| Average dry wt. | 7.4 | 17.5 | 33.3 |

* Adaptation.

ture, they were called complete adaptations if their weights were very similar to those of the wild-type mold, or partial adaptations if their weights were

intermediate. In the assay procedure these weights were ruled out by an arbitrary statistical means. All cultures whose weights were higher than the average of the remainder of a series by more than $2\sigma$ (standard deviations) were classed as adapted. In the studies to be reported, $3\sigma$ was used to identify adapted cultures.

When samples were taken from adapted cultures and transferred to medium devoid of leucine, growth continued to take place. Repeated asexual subculturing on such minimal medium always resulted in leucine-independent growth. The leucine-independence was inherited, therefore, during asexual vegetative reproduction. Since strains originating through adaptation behave in their nutritional requirements like the "wild-type" Neurospora from which the deficient mutants were derived, they may be called prototrophic. This term has no genetic or teleological implications.

The adapted mold is, so far as we can tell, physiologically identical with "wild-type" strains (*25*). In the absence of leucine, not only the rate of growth but also the amount of mycelium finally produced is the same. The addition of various amounts of leucine neither stimulates nor retards the growth of either adapted or "wild-type" strains. The leucine content of both, as determined by the Neurospora assay procedure, is also identical (*ca.* 2.3%). The possibility that adaptation involves the production of a physiologically active substitute for leucine is excluded by the chemical demonstration of $l(+)$-leucine in a hydrolysate of adapted mycelium. This was achieved by means of a solubility product test of the $l(+)$-leucine bromotoluene sulfonate salt (*19*). Hence, the adapted mold has regained the ability to synthesize $l(+)$-leucine, and consequently it no longer requires leucine in the medium. It has become leucine-independent.

The probability that contamination was responsible for the origin of adaptation is extremely slight, because genetic markers for color and for mating type were not modified during adaptation. There remain three reasonable explanations of the occurrence of adaptations in leucineless Neurospora. The first hypothesis assumes a cytoplasmic learning to make leucine in its absence, due, for example, to the assumption of an alternate synthetic pathway (*31*). In such a nongenic case the changed situation would not be expected to segregate in a Mendelian fashion. The second hypothesis assumes a mutation at some locus other than the leucineless locus ($l_1$) in strain 33757. This mutation would suppress $l_1$ and, of course, would at times segregate from it during meiosis. The third interpretation assumes a back-mutation of the $l_1$ factor to the wild-type condition, $L$.

These hypotheses were tested by crossing adapted cultures with a leucineless strain of the opposite mating type (*25*). Asci produced from such a cross contained both prototrophic leucine-independent and leucineless spores in a 1:1 ratio. Hence, the first hypothesis can be discarded; adaptation involves a genic mutation. In order to discriminate between the last two hypotheses, crosses were made between the prototrophic $F_1$ strains secured from spores from the last cross and "wild-type."

$$P_1 \quad \text{adapted} \times \text{leucineless}$$

$$F_1 \quad \text{4 leucineless: 4 prototrophic} \times \text{"wild-type"}$$
spores per ascus

8 prototrophic spores per ascus

If the factor for leucine-independence were at a different locus than $l_1$, then the recombination class of leucineless should appear among the progeny. No such leucineless strains were secured among sufficient tests to indicate that if the two factors were nonallelic they must be within a few map units of each other on the same chromosome. It is not experimentally possible to distinguish a back-mutation from a suppressor mutation in one of a series of adjacent leucineless genes. Such blocks of physiologically identical or similar genes seem a real possibility. Nevertheless, in the present state of our knowledge the simplest hypothesis consistent with both the genetic and the physiological data is that adaptation of leucineless Neurospora is due to the reverse-mutation of the leucineless factor, $l_1$, to its "wild-type" condition, $L$.

The possibility of a back-mutation is indirect evidence for the self-duplication of the leucineless factor $l_1$. Presumably the effect of ultraviolet light on $L$ was to produce a situation, $l_1$, incapable of activity in the synthesis of leucine, yet capable of reproduction and capable of reverting to the original form. The $l_1$ condition could not very likely be a large chromosomal deficiency. It could possibly be a position effect resulting from the transposition of the $L$ locus to a new location. However, our attempts to find evidence of chromosomal rearrangements have not succeeded. They have involved examination of asci for spore abnormalities and a still incomplete cytological investigation.

In the absence of positive evidence for a gross chromosomal rearrangement we tentatively conclude that $l_1$ differs from $L$ in a chemical change in

TABLE 2. THE EFFECT OF pH ON THE FREQUENCY OF ADAPTATIONS OF THE NEUROSPORA LEUCINELESS STRAIN 33757–4637-A DURING 8½ DAYS IN 50 ML. OF LIQUID MEDIUM AT 30° C.

| pH | Number of cultures | Number of adaptations | Per cent adaptation |
|-----|-----|-----|-----|
| 4.9 | 28 | 7 | 25 |
| 5.5 | 28 | 10 | 36 |
| 6.0 | 27 | 6 | 22 |
| 6.5 | 27 | 4 | 15 |

a restricted region of a chromosome. We must bear in mind that in the absence of specific and detailed knowledge of the nature of a mutated gene, or even of any gene, it is difficult conceptually to draw the line between, say, the duplication of groups or of molecules and the duplication of visible regions of a chromosome.

TABLE 3. THE EFFECT OF TEMPERATURE ON THE FREQUENCY OF ADAPTATIONS OF THE LEUCINELESS NEUROSPORA STRAIN 33757-4637-A DURING 8½ DAYS IN 50 ML. OF LIQUID MEDIUM

| Temperature °C. | Number of cultures | Number of adaptations | Per cent adaptation |
|---|---|---|---|
| 35 | 79 | 11 | 14 |
| 30 | 80 | 11 | 14 |
| 25 | 160 | 4 | 3 |
| 20 | 148 | 8 | 5 |
| 15 | 79 | 4 | 5 |

The frequency of adaptation of strain 33757-4637-A of Neurospora is not independent of environmental factors. Table 2 shows that pH is without effect on adaptation at 30° C. in the presence of 0.21 mg. of $l(+)$-leucine per ml. The pH of Fries medium is normally about 5.5. The differences in frequency of adaptation shown in Table 2 are not significant by $\chi^2$ test. On the other hand, temperature has a pronounced effect, as Table 3 shows. At each temperature there were approximately equal numbers of cultures grown on 0.25, 0.50, 0.75, and 1.00 mg. $l(+)$-leucine per 50 ml. The data for 35° may not be strictly comparable with other entries in this table, for this temperature is above the optimum for growth of strain 33757-4637-A on low-leucine concentrations and the mycelium is morphologically very different. Nevertheless, the per cent adaptation at this temperature and at 30°, where growth is normal, is significantly greater by $\chi^2$ test than the per cent adaptation at the lower temperatures. Among the lower temperatures, 15-25°, there seems to be a constant adaptation frequency.

Table 4 shows the effect of leucine concentration on the adaptation frequency of strain 33757-

4637-A, which data are summarized from Ryan and Lederberg (25). On a spontaneous-back-mutation hypothesis one would expect the back-mutation frequency to be greater in the cultures containing large amounts of leucine, for it is there that larger mycelial masses with greater nuclear populations are formed. The reverse is true, however; adaptation
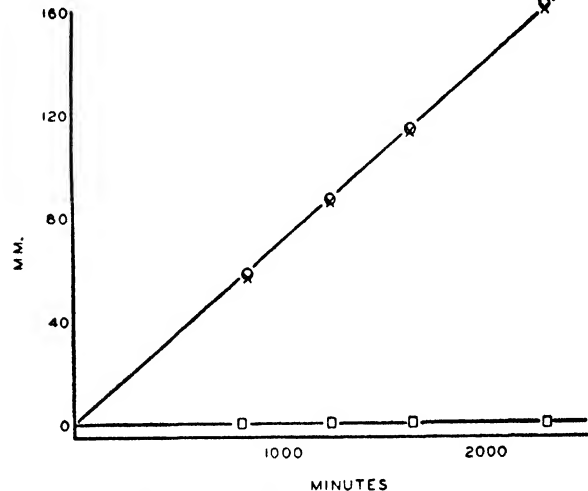


FIG. 2. The growth rate of a heterocaryon composed of a leucineless and a leucine-independent strain of Neurospora, and the rates of the separate components in growth tubes on minimal agar medium at 25° C. Squares represent the leucineless strain, x's the leucine-independent, and circles the heterocaryon.

frequency varies inversely with the amount of leucine present. Such a relation between adaptation and the leucine content of the medium seems to represent an example of chemically induced mutation. Before this conclusion can be accepted, however, consideration must be given to the possibility of competition between nuclei in a heterocaryon.

When a back-mutation from the leucineless to the leucine-independent condition occurs in Neurospora a heterocaryon is formed containing leucineless and prototrophic nuclei. Such heterocaryons have been prepared artificially and their growth

TABLE 4. THE EFFECT OF LEUCINE CONCENTRATION ON THE FREQUENCY OF ADAPTATIONS OF THE LEUCINELESS NEUROSPORA STRAIN 33757-4637-A IN 50 ML. OF LIQUID MEDIUM

| Mg. $l(+)$-leucine | During 8½ days after inoculation; data from 25° and 30° C. combined | | | Between 6 and 45 days after inoculation, 30° C. | | |
|---|---|---|---|---|---|---|
| | Number of cultures | Number of adaptations | Per cent adaptation | Number of cultures | Number of adaptations | Per cent adaptation |
| 1.00 | 60 | 0 | 0 | — | — | — |
| 0.75 | 60 | 4 | 7 | — | — | — |
| 0.50 | 60 | 3 | 5 | 43 | 5 | 12 |
| 0.25 | 60 | 8 | 13 | 38 | 16 | 42 |

characteristics and nuclear composition have been studied (25). Proof that heterocaryons were actually formed in these experiments comes from the isolation of hyphal tips in which both leucineless and leucine-independent nuclei could be demonstrated. When these heterocaryons were grown on minimal agar medium a behavior like that shown in Fig. 2 was obtained. The "wild-type" component by itself grew at a rate of about 4.4 mm./hr. while the leucineless component did not grow at all. The heterocaryon grew at the same rate as the "wild-type." On a limiting concentration of leucine the "wild-type" still grew at a rate of 4.4 mm./hr. while the leucineless mold grew at a rate of 2.9 mm./hr. (Fig. 3). The point of interest here is that

functional alleles in other nuclei (Fig. 4). When a heterocaryon was formed between a leucineless strain marked with albino-1 and a leucine-independent strain marked with albino-2, pigment was formed in the conidia. These colored heterocaryons



FIG. 4. Diagram of the probable relationships in a heterocaryon composed of strains of Neurospora carrying different genes for albinism.

exhibited the same type of selection shown in Figs. 1 and 2. Moreover, the conidia formed at the far end of a growth tube containing a limiting concentration of leucine were white. When tested on minimal medium they proved to be leucineless, and the color marker of the leucine-independent nuclei could not be demonstrated by making heterocaryons with the original albino strains. On minimal medium the conidia at the far end of the tube were also white but proved on test to be leucine-independent, and the color marker of the leucineless nuclei could not be demonstrated. Consequently, it can be concluded that the selection observed in these heterocaryons involves the functional elimination of one type of nucleus.



FIG. 3. The growth rate of a heterocaryon composed of a leucineless and a leucine-independent strain of Neurospora, and the rates of the separate components in growth tubes on agar medium containing a limiting concentration of $l(+)$-leucine (0.0158 mg. $l(+)$-leucine/ml.). Squares represent the leucineless strain, x's the leucine-independent, and circles the heterocaryon.

the heterocaryon, although it contained a "wild-type" component, grew at the slow rate of the leucineless strain. Apparently, in the presence of leucine the leucineless nuclei in the heterocaryon have a selective advantage over leucine-independent nuclei and are selected for.

Evidence that this selection is not a simple transformation of the $L$ locus to an inactive state comes from the demonstration of (at least) the functional loss of the whole prototrophic nucleus. In proving this, advantage was taken of the fact that there are two albino mutants of Neurospora which when grown together in a heterocaryon form pigment (1). This is presumably due to the fact that a heterocaryon between strains differing in different mutant alleles is physiologically similar to a heterozygous diploid organism. Mutant genes are covered by

It is not known whether the unsuccessful leucine-independent nuclei are inhibited or actually destroyed in the presence of leucine or whether they are simply left behind during the movement of nuclei with the streaming cytoplasm into the developing hyphal tips. Attempts to demonstrate an extractable inhibitor of leucine-independent nuclei from both leucineless strains and heterocaryons have been unsuccessful (Lederberg, unpublished). It is known, however, that the selection is a function of the difference between $L$ and $l_1$ in the strains forming the heterocaryon, for it occurs in heterocaryons made between a leucineless strain and an adapted strain derived from it. It is unlikely that two such strains would differ genetically in more than these alleles. It is also probable that the genetic background can influence this selection, for there are some strains carrying a leucine-independent gene (which may not be the same allele as $L$) that do not show this selection in heterocaryons with leucineless either on minimal medium or in the presence of leucine. The fact that even selection for the prototrophic

nuclei does not take place on minimal medium in such stable heterocaryons may indicate that inactivation of the prototrophic nuclei by the leucineless is not due to the production of a chemical inhibitor.

Selection in heterocaryons where it is capable of occurring is not a completely predictable event. On agar medium with a limiting leucine concentration it may be so rapid that the heterocaryon grows from the start like leucineless Neurospora. Or a period of growth at the wild-type rate may be passed through before the leucineless nuclei are selected for and growth slows down. Occasionally, a labile heterocaryon on limiting leucine will fail to select to a leucineless condition. Or, after the rate of growth has begun to slow down and to approach that of the leucineless mold, it may suddenly speed up as though the few remaining prototrophic nuclei have suddenly escaped in some way from the influence of the leucineless nuclei. On the other hand, a labile heterocaryon on minimal medium devoid of leucine may sometimes grow for a while at the rate of "wild-type" Neurospora and then slow down and completely stop. It would seem as though a few active leucineless nuclei were carried along as the frontier progressed at "wild-type" rate but eventually came to dominate the hyphal tips and prevented further growth. It is possible, by a consideration of the selection phenomenon and the aspect of chance involved, to provide a different interpretation of the frequency of adaptations on different concentrations of leucine than that of a chemical induction of mutation.

The selection phenomenon enables us to understand how leucine has its effect on adaptation in liquid medium through the *mass* of mycelium whose growth it supports. There are three types of experiment which show that adaptations can occur more readily in small masses of mycelium than in large and hence, presumably, in the presence of small numbers of leucineless nuclei more readily than in the presence of large numbers. In the first place, when the leucineless strain is grown in the presence of limiting amounts of leucine, at the completion of growth the medium is exhausted of that compound. Sterile filtration and reinoculation with the leucineless mold results in no further growth even if additional sterile sugar is added to the medium. Only if new sterile leucine is added can growth of the leucineless mold be made to resume, and then it occurs to the extent expected on the basis of the leucine added. Two series of cultures were observed in the presence of subthreshold concentrations of leucine at 30° C. for 39 days after the final dry weights of about 7 and 16 mg. had been attained. The small mycelial masses showed 42% adaptation while the large masses showed only 12% (see Table 4).

Although it is impossible to duplicate accurately the introduction of a prototrophic nucleus into a leucineless mycelium, which occurs upon back-mu-

tation, it is possible to introduce small pieces of adapted mycelium into clots of leucineless mycelium of different sizes. Cultures of an adapted strain, β, were grown on minimal medium to a dry weight of 0.3 mg. They were then inserted into clots of mycelium of leucineless strain 33757-4637-A, which had grown for 8½ days to completion at 25° C. on 0.24 and 0.97 mg. of $l(+)$-leucine per 50 ml. and which had dry weights of about 7 and 37 mg. The

TABLE 5. The Final Weight Produced at 25° C. after the Insertion of Small Pieces of an Adapted Strain into the Mycelium of the Neurospora Leucineless Strain 33757–4637-A which had Stopped Growing

| Initial concentration of $l(+)$-leucine, mg./50 ml. | 0.24 | 0.97 |
|---|---|---|
| Approximate dry weight of leucineless strain at insertion, mg. | 7 | 37 |
| Mg. dry weights of 40 different cultures 8½ days after insertion | 92.1 | 62.8 |
| | 98.8 | 58.6 |
| | 87.9 | 37.5* |
| | 88.8 | 61.1 |
| | 82.9 | 60.8 |
| | 87.2 | 62.4 |
| | 8.8* | 65.7 |
| | 85.5 | 59.9 |
| | 94.6 | 60.8 |
| | 85.1 | 58.6 |
| | 88.4 | 61.9 |
| | 90.5 | 56.7 |
| | 85.2 | 60.5 |
| | 74.8* | 58.1 |
| | 16.0* | 56.0 |
| | 90.2 | 59.3 |
| | 59.5* | 61.0 |
| | 89.1 | 59.4 |
| | 92.1 | 64.9 |
| | 89.5 | 62.1 |

* See text.

additional weight of such cultures, shown in Table 5, is, of course, a consequence of the growth of the adapted strain. Insertion into the small mycelia resulted in more growth than insertion into the large mycelia. Neither series attained the *ca.*-110 mg. weights that β can assume by itself on this medium. Apparently the larger mass of mycelium was more efficient in preventing the growth of the prototrophic mycelium than the smaller mass. In both cases heterocaryons were undoubtedly formed. Nevertheless, selection was not uniform, for, as the weights marked with asterisks in Table 5 show, sometimes dominance of the leucineless mycelium was more efficient than usual.

When heterocaryons between adapted and leucineless strains were prepared on agar slants and then inoculated into liquid medium containing different amounts of leucine, results yielding the same interpretation were obtained. Fig. 5 shows the behavior of such heterocaryons and of their component strains. The weight of the adapted strain was

independent of the amount of leucine present. The leucineless strain formed a mycelium whose weight was proportional to the amount of leucine present. The heterocaryon, on the other hand, yielded weights that decreased with increasing amounts of leucine and then increased when the weights became similar to those of the leucineless mold. Apparently
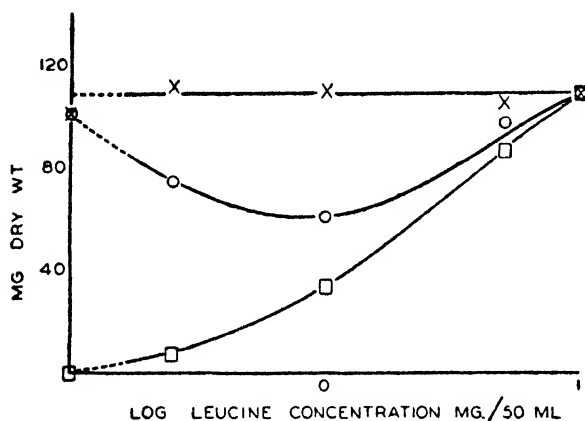


FIG. 5. Final growth of a heterocaryon composed of leucineless and adapted Neurospora, and of the separate components, after 8½ days at 25° C. in 50 ml. of liquid medium containing different concentrations of $l(+)$-leucine. Squares represent leucineless, x's the adapted mold, and circles the heterocaryon.

in a heterocaryon the larger the number of leucineless nuclei whose growth is supported by the leucine present, the less growth the prototrophic nuclei can undergo. This relation holds until the growth produced by the heterocaryon is almost completely composed of the leucineless component, which seems to be the case in the presence of large amounts of leucine.

With these facts available, we can suppose that the influence of leucine on adaptation rate is not through the chemical induction of mutations but rather through its influence on the mass of leucineless mycelium. The larger the mass the less is the chance that a back-mutated nucleus of "spontaneous" origin will escape from the influence of leucineless nuclei and form an adaptation. Actually, in stationary cultures at maximum weight there sometimes appear new patches of growth. If these continue to grow a complete adaptation is formed. On most occasions, owing to the action of the leucineless nuclei, the new spot of growth stops growing and the culture becomes a partial adaptation. It must be emphasized that although our interpretation undoubtedly describes some of the events taking place during and after back-mutation it does not exclude the possibility that leucine or its absence may play, in addition, a direct chemical role in the induction of mutation. In the absence of selection and on a spontaneous-mutation hypothesis, about twice as many back-mutations would be

expected to occur in a mycelium twice as large as another. And yet we find about four times as many adaptations among mycelia half the size of others. This is an eightfold difference from expectation. On this basis we can assume that the vast majority of back-mutations do not express themselves. On account of the selection phenomenon, then, back-mutation of the leucineless strain 33757-4637-A of Neurospora is not a favorable object for the study of mutation rate and, hence, of the physiology of mutation itself. The influence of temperature shown in Table 3, for example, may be on the rate of back-mutation, on selection, or on both processes.

TABLE 6. THE FREQUENCY OF ADAPTATION OF THE LEUCINE-LESS NEUROSPORA STRAIN 33757-a DURING 8½ DAYS IN 50 ML. OF LIQUID MEDIUM AT 30° C.

| Mg. $l(+)$-leucine | Number of cultures | Number of adaptations | Per cent adaptation |
|---|---|---|---|
| 1.06 | 10 | 1 | 10 |
| 0.79 | 10 | 0 | 0 |
| 0.53 | 10 | 0 | 0 |
| 0.26 | 10 | 3 | 30 |
| Average % adaptation | | | 10 |

The adaptation frequency of another strain of Neurospora containing the mutant leucineless gene, $l_1$, is not essentially different from that of strain 33757-4637-A. Table 6 shows the adaptation of strain 33757-a. Apparently whatever differences exist in the genetic background have not influenced either the mutation rate or the selection efficiency.

There is another Neurospora leucineless strain, 8839-4637-A, carrying a mutant gene which is not

TABLE 7. THE FREQUENCY OF ADAPTATION OF THE LEUCINE-LESS NEUROSPORA STRAIN 8839-4637-A DURING 8½ DAYS IN 50 ML. OF LIQUID MEDIUM AT 30° C.

| Mg. $l(+)$-leucine | Number of cultures | Number of adaptations | Per cent adaptation |
|---|---|---|---|
| 1.00 | 15 | 2 | 13 |
| 0.75 | 17 | 0 | 0 |
| 0.50 | 18 | 1 | 6 |
| 0.25 | 15 | 4 | 27 |
| Average % adaptation | | | 11 |

allelic with $L$ and $l_1$ but is concerned with a different step in the synthesis of leucine. Although on minimal medium neither strain 8839-4637-A nor strain 33757-4637-A by itself will grow at all, when they are together in a heterocaryon vigorous growth is achieved. Mr. T. C. Sheng, in our laboratory, has crossed 8839-4637-A with 33757-a and has evidence that 8839 behaves as a single factor, which we may call $l_1'$, segregating from the leucine-independent allele $L'$. Strain 8839-4637-A undergoes adaptation at 30° with a frequency similar to that of strain 33757-4637-A (Table 7). Although such adapta-

tions can be maintained vegetatively in the absence of leucine, it has not been proved whether they are the result of back-mutation of $l_1'$ to $L'$. However, in heterocaryons the same type of selection between $l_1'$ and $L'$ takes place as occurs between $l_1$ and $L$. Figs. 2 and 3 could represent the behavior of heterocaryons between strain 8839-4637-A and a prototrophic strain derived from it. Indeed, Figs. 2 and 3 were actually drawn from data collected by Mr. Sheng on a heterocaryon between a double leucineless mutant, 33757-8839-B8, obtained by breeding, and a leucine-independent strain, 15300-a. The selection phenomenon is essentially the same in the double mutant as in the two single leucineless mutants *(25)*.

The adaptation frequency of the double mutant is close to zero (Table 8). On the basis of the known mutation frequencies of its two components,

TABLE 8. THE FREQUENCY OF ADAPTATION OF THE NEURO-SPORA DOUBLE LEUCINELESS MUTANT 33757–4637-B8 DURING 8½ DAYS IN 50 ML. OF LIQUID MEDIUM AT 30° C.

| Mg. $l(+)$-leucine | Number of cultures | Number of adaptations | Per cent adaptation |
|---|---|---|---|
| 1.00 | 14 | 0 | 0 |
| 0.75 | 15 | 0 | 0 |
| 0.50 | 15 | 0 | 0 |
| 0.25 | 15 | 0 | 0 |
| Average % adaptation | | | 0 |

it might be expected to be considerably less than 1%.

Some other mutants of Neurospora, which have single deficiencies, are also very stable. The lysineless strain, 4545, and the pantothenicless strain, 5531, have never adapted in our hands although they have been extensively cultured. Others, however, frequently undergo adaptation although in many cases—such as that of the *p*-aminobenzoicless mutant, 1633, *(40, 2)*—it is not known whether the adaptation is even inherited asexually. In some unpublished instances the adaptations are known to be genic in their origin. There are also instances, however, where the adaptations are not inherited, as in the cases of the adaptation of the *p*-aminobenzoicless strain 1633 to use pimelic acid *(34)*, of the isoleucine-valine mutant 16117 to dispense with these substances *(4; Bonner and Tatum, private communication)*, and of the leucineless strain 33757 to use leucic acid *(20)*. Two more cases of noninherited adaptation may be mentioned. The prolineless mutant of Neurospora, 21863, when grown in 50 ml. of liquid medium at 30° yields the relationship to $l(-)$-proline shown in Fig. 6. In the higher proline concentrations many of the cultures have weights considerably higher than the smallest. These heavy cultures appear to be similar to adaptations in the leucineless strain. But, unlike leucineless adaptations, these prolineless "adaptations" are

not inherited vegetatively. Transfers of bits of mycelium, which were raised in the presence of 0.8 mg. $l(-)$-proline per 50 ml., to proline-free medium showed no growth in 26 attempts. And yet the mycelia from which subcultures were attempted weighed between 29 and 42 mg., so that it is unlikely that all of the samples were taken from prolineless regions of a clot which elsewhere had undergone
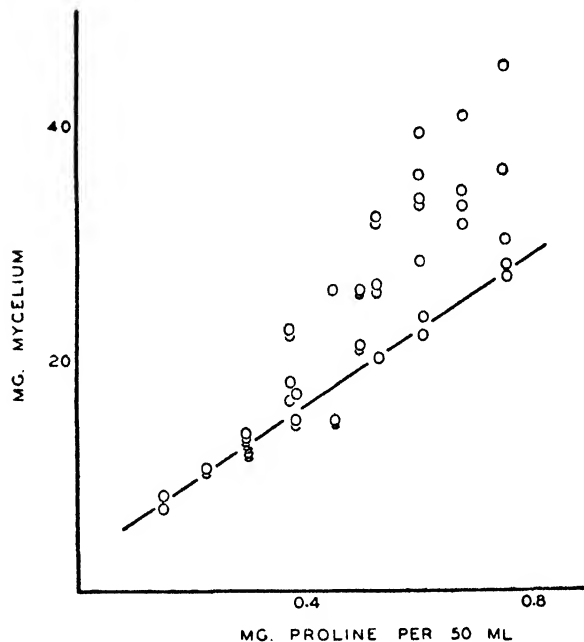


FIG. 6. The relation of proline to the dry weight of mycelium produced by the prolineless Neurospora in 50 ml. of liquid medium after 8½ days at 30° C.

an inheritable adaptation. Another instance is the slow adaptation of the Neurospora thiaminless mutant, 9185. On the surface of thiamin-free agar this mutant may not grow for several days, but then it slowly begins to grow and eventually progresses at the wild-type rate. This adaptation is not inherited through the conidia. There are also several instances of adaptive enzyme formation known in Neurospora *(10, 30, 35)*.

Adaptations of the Neurospora mutants have not been studied sufficiently to realize all of the possibilities. It may well be that both genic and nongenic adaptations may occur in the same strain. However, our studies on the leucineless mutants provide ample reason for the constant consideration of nuclear selection in studies on adaptation or back mutation. This admonition applies not only to investigations on heterocaryon-formers like Neurospora, but also to studies of mutation in any population of growing organisms, such as bacteria.

The nutritional requirements of bacteria are not always stable but may be gained or lost either "spontaneously" or owing to experimental manipulation *(18)*. The rate of loss of requirement may

be rapid or slow, depending upon both the organism and the requirement. We have studied the adaptation to uracil-independence of strain 59 Li of *Clostridium septicum* (22).

This strain will grow in a chemically defined medium which contains no purines or pyrimidines, but in the erratic and inconsistent way shown in Table 9. But when growth does occur it is always at the same logarithmic rate and always yields the same amount of bacterial nitrogen. It was found that the variability in the onset of growth could be eliminated by the addition of the pyrimidine,

TABLE 9. VARIATION IN THE ONSET OF GROWTH OF STRAIN 59 LI OF CLOSTRIDIUM SEPTICUM ON A CHEMICALLY DEFINED MEDIUM (RYAN ET AL., 1946)

| Tube | ꝼ Appearance of cultures at various hours after inoculation | | | |
|------|-----|-----|-----|-----|
|      | *8* | *22* | *24* | *51* |
| 1  | − | − | − | − |
| 2  | − | − | + | +++ |
| 3  | − | − | + | +++ |
| 4  | − | − | − | − |
| 5  | − | + | +++ | +++ |
| 6  | − | +++ | +++ | +++ |
| 7  | − | + | +++ | +++ |
| 8  | − | +++ | +++ | +++ |
| 9  | − | + | +++ | +++ |
| 10 | − | − | + | +++ |
| 11 | − | − | − | − |
| 12 | − | − | ++ | +++ |

uracil, to the medium. This significantly reduced the lag period (Table 10) but did not affect either the rate of growth during the logarithmic phase or the size of the final crop of bacteria. Its effect was solely in diminishing the length of the lag period and in assuring growth.

As an explanation of this phenomenon we can

TABLE 10. TIME IN HOURS FOR STRAIN 59 LI TO REACH +++ GROWTH IN A CHEMICALLY DEFINED MEDIUM SUPPLEMENTED WITH PURINES AND PYRIMIDINES (RYAN ET AL., 1946)

| | |
|---|---|
| No supplement | 43 |
| Adenine | 55 |
| Guanine | 43 |
| Xanthine | 30 |
| Uracil | 21 |

assume that strain 59 Li consists of two types of organism, one type requiring an external supply of uracil for growth and another type which synthesizes uracil and does not require it in the medium. If, by chance, there are no uracil-independent cells in the inoculum, growth will not ensue in a medium devoid of uracil. If the number of uracil-independent organisms is small, growth will take place after a long lag period; if large, it will occur rapidly.

Verification of the general aspects of this hy-

pothesis calls for a demonstration of the two types of bacteria. A culture of strain 59 Li was plated on blood agar and single colonies were isolated. From these colonies both uracil-dependent and uracil-independent strains were secured. The response of two of the new strains to uracil is shown in Table 11. The dependent strains behaved like the parent strain and showed, in the absence of uracil, a long and variable lag period. The 1630 minutes until growth equivalent to 2 mg. of nitrogen shown in Table 11 for the dependent strain, B, is an average based upon six cultures from two different experi-

TABLE 11. THE EFFECT OF URACIL ON THE GROWTH OF URACIL-DEPENDENT AND URACIL-INDEPENDENT STRAINS OF CLOSTRIDIUM SEPTICUM (RYAN ET AL., 1946)

| | Dependent—B | | Independent—A | |
|---|---|---|---|---|
| Uracil γ/ml. | 0 | 10 | 0 | 10 |
| Minutes to 2 mg. bacterial nitrogen | 1630±500 | 800±162 | 690±40 | 739±40 |
| Generation time during log. phase in min. | 79 | 78 | 59 | 62 |
| Mg. bacterial nitrogen after 48 hours | 12.0 | 12.5 | 14.9 | 14.5 |

ments. Not included in the average were 4 cultures that began to grow between the third and fifth day after inoculation and 4 which had not grown by the sixth day. The presence of uracil, however, caused the dependent strain to grow rapidly and regularly. The independent strain, A, grew up rapidly and regularly, irrespective of the presence of uracil. Thus, both independent and dependent cells are present in a culture of strain 59 Li.

The new strains, A and B, were themselves plated onto blood agar, and single colonies were isolated. Not only did the new dependent strain, B, again give rise to both dependent and independent types, but the new independent strain, A, did likewise. Since care was taken to isolate well-separated colonies in single spherical zones of hemolysis on the blood-agar plates, since *all* of the dependent strains developed uracil-independence as was shown by their eventual growth in the absence of uracil, and since microscopic observation showed that agar did not cause a clumping of bacteria, it is unlikely that any of the isolated colonies arose from more than one organism. Therefore the dual composition of the dependent and independent strains must have resulted from the mutation of one type to the other.

The ability of the dependent organisms to give rise to independent ones suggests that some of the variation in the onset of growth of the dependent strain on uracil-free medium may be due to such mutation. This would account for the sudden growth which sometimes occurs in cultures that have remained dormant for as long as 5 days. In an or-

ganism which has a logarithmic generation time of about 80 minutes and in which only about 10 generations need occur between the inoculum and final growth, 5 days seems an unreasonably long lag period on any other basis. However, we have not been able to demonstrate that mutation occurs to uracil-independence in a dependent strain suspended in a nongrowing condition in uracil-free medium. When a washed inoculum containing about $10^6$ cells was used, growth of the dependent strain took place equally well in the absence or in the presence of uracil. Apparently the inoculum contained an absolute number of uracil-independent organisms large enough to permit rapid growth. Dependent inocula containing between $10^3$ and $10^5$ cells show the variable growth in the absence of uracil which has already been described. The inoculum size we have routinely employed is about $10^3$. When the inoculum size is as small as $10^2$, growth fails to occur not only in the absence of uracil but also in its presence. Since we found no inoculum size at which growth occurred in the presence of uracil but regularly did not take place in its absence, it is probable that the variable growth of a dependent strain when inoculated into uracil-free medium is usually a consequence of a variable content of independent cells already present in the inoculum.

Determinations of the likely average proportion of independent organisms, however, give the surprisingly high value of 75%, with the likely variation between 50 and 100%. In view of this, it becomes difficult to understand how the number of independent organisms in dependent inocula containing a total of about $10^3$ cells would vary sufficiently to account for the observed variation in the onset of growth in uracil-free medium, especially when the lag may sometimes be as long as 5 days. But recourse may be had to the fact that a very small inoculum will not grow even in the presence of uracil. We may be dealing with a situation where the loss of some essential substrate occurs too rapidly in a dilute suspension. At any rate, our usual inoculum size of $10^3$ organisms may be too close to the minimum size, so that occasionally we do not provide enough uracil-independent organisms for growth to occur. At other times a marginal number of independent organisms in the inoculum may recover and grow. On the other hand, in rare instances, even with an inoculum of about $10^3$ cells, the dependent strain will grow up rapidly in all of 10 tubes containing uracil-free medium, although upon further test the strain will prove to be dependent. Presumably in such cases the culture from which the inoculum was taken contained close to 100% of independent cells. We therefore conclude that several factors may be responsible for the variation in the onset of growth of the dependent strain in uracil-free medium. Although the proportion of independent cells and, perhaps, mutation to

uracil-independence are involved, the major factor is probably the absolute number of uracil-independent cells in the inoculum.

Advantage can be taken of the variation in proportion of uracil-independent organisms from culture to culture to discriminate between the "spontaneity" of these mutations and their induction by the testing technique. The basis of the procedure has been described by Luria and Delbrück (7, 17). When it is applied to organisms mutating to a complete requirement for some growth factor, only mutations in the presence of that growth factor can be studied. However, the rate of mutation from uracil-independence to uracil-dependence is relatively slow. When platings were made soon after the independent strain, A, had been isolated, no uracil-dependent cells could be identified. When platings were made from the same stock tube several months after isolation, however, 1 out of 41 colonies contained uracil-dependent cells. An independent determination at this time indicated that about 98% of the cells were still uracil-independent. Since the stock tube contained uracil, however, it is probable that mutation occurred in its presence. On the other hand, mutation from dependence to independence may be very rapid. Dependent cultures derived from freshly isolated colonies contain a majority of independent cells. We know nothing of the role of selection in this phenomenon, although by itself one independent strain grew faster and farther than a dependent strain (Table 11). The study of mutation to uracil-independence must also be made in medium containing uracil.

Ten 2-ml. lots of chemically defined medium containing uracil were each inoculated with about $10^3$ cells of a uracil-dependent strain of *Clostridium septicum*. After growth was complete, the number of uracil-independent cells was determined by taking one sample from each of 9 tubes and 9 samples from the 10th tube. There was a 0.24 probability that the distribution of proportions of independent bacteria among the 9 samples taken from a single tube was due to chance. On the other hand, there was only a 0.007 probability that the distribution of proportions of independent bacteria among the samples from the 9 separate tubes was due to chance. Since this distribution was probably not due to sampling, it may have been due to the random occurrence of mutations to uracil-independence during growth of the uracil-dependent culture in the presence of uracil. Early-occurring mutations would have a greater effect on the final proportion of uracil-independent organisms than those occurring late. However, in the case of a dependent strain of *Clostridium septicum*, where so many independent cells are already present in the inoculum, the variation is very much less than that observed in other instances (8, 9, 17, 36).

Our chemically defined medium contains gluta-

mine, but when glutamine is omitted the same type of irregularity in growth occurs as in uracil-free medium. It may be that there are also glutamine-dependent and glutamine-independent strains of *Clostridium septicum*, which arise from one another by mutation. But because the anaerobic, pathogenic, gas-forming *Clostridium septicum* is very difficult to work with, our studies were transferred to the nutritional mutants of *Escherichia coli* (*11, 32*), where adaptations are also known to occur (*21*). Through the courtesy of Drs. E. L. Tatum and J. O. Lampen we have secured X-ray-induced mutants of *E. coli* each of which requires one or more amino acids for growth. Our studies on these mutants are only preliminary, and the results and their interpretation should be considered in this light.

When the nutritional mutants of *E. coli* are inoculated into minimal medium or into medium containing limiting amounts of the missing growth factor, complete growth of the cultures will eventually occur (*21*). Likewise, when cultures of the amino-acid mutants are heavily plated onto amino-acid-free minimal agar medium, a number of colonies appear which consist of organisms that have lost their amino-acid requirements. The number of such reverted colonies varies considerably from one mutant strain to another. Some strains, like Roepke's methionineless 532-171, are very stable; while others, like Lampen's lysineless 15L-171, are very unstable. Lampen's histidineless strain 148-334 is intermediate in stability. One ml. of a culture grown on medium containing histidine and possessing about $10^9$ bacteria contains about 100 histidine-independent organisms per ml., as determined by plating on minimal agar. These reverted deep-agar colonies are not contaminants, for they give rise to strains which are prototrophic but which grow at exactly the same rate as the original histidineless mutant in medium supplemented with an optimum amount of histidine. There is apparently no strong competition between the histidineless mutant and the adapted strain derived from it, for when a mixture of these two strains in approximately equal proportions is inoculated into a fresh tube of medium containing histidine the proportion of both types remains the same.

The appearance of mutations to histidine-independence in medium containing histidine may be due to a phenomenon which has a given chance of occurring per organism per unit time throughout the growth of a histidineless culture. Comparisons of the variation in number of prototrophic organisms among different cultures with the variation found in different samples from the same tube indicate an insignificant chance ($<0.001$) that the variation from culture to culture is due to sampling. The back-mutation rate is of the order of $10^{-8}$. It is possible, however, that these mutations are not random, although they may occur at different times

during growth. Some organisms in a culture may be generally more mutable than others.

Tatum has obtained a double mutant, 679-680, which requires both threonine and leucine for growth, by the further irradiation of a threonineless mutant (*32*). Mutations to threonine-independence can be detected on medium containing leucine but not threonine, while on medium containing threonine we can detect mutations to leucine-independence. When 50 washed cultures grown in the presence of leucine and threonine were heavily plated onto agar containing leucine but not threonine and onto agar containing threonine but not leucine, a small number of colonies appeared. The threonine-independent or leucine-independent organisms that formed these colonies occurred each with a frequency of about $10^{-8}$. The number of threonine-independent cells in the original 50 cultures did not seem to be correlated with the number of leucine-independent cells. The number of double reversions on minimal agar would be more meaningful. The frequency with which these occur is not yet certain.

There have been many instances of the adaptation of strains of bacteria and yeasts to dispense with complete growth-factor requirements (*12, 13, 14, 15, 16, 18, 26, 27, 28, 29, 37, 38*). The principles of the techniques employed (heavy inoculum and serial transfer in the presence of limiting amounts of growth factor) are such as to suggest that the adaptations may have been caused by the selection of mutants. However, such a generalization on the basis of the data already presented would probably be unjustified. Each case should be decided on its own merits after examination with the techniques now available; and in each case the possible role of selection must be determined by examination of the behavior of mutant and back-mutated organisms *together*. Selection phenomena similar to that occurring between leucineless and prototrophic nuclei in Neurospora may also occur in bacteria. For example, Wright and Skeggs (*39*) have selected a tryptophane-independent strain of *Lactobacillus arabinosus* after serial transfer in tryptophane-deficient medium. When this strain is cultured in the presence of increasing amounts of tryptophane, its growth is progressively depressed until it reaches a level equal to that of the tryptophaneless parent when it similarly rises. This relationship (*39*, Fig. 3) is identical with that shown in Fig. 4 for the heterocaryon of leucineless and prototrophic Neurospora. It suggests a similar competition between tryptophaneless and tryptophane-independent *L. arabinosus* in the presence of tryptophane.

Our attempts to secure a tryptophane-independent strain of *L. arabinosus* by massive inocula into tryptophane-free liquid and agar media have failed. The success that Wright and Skeggs (*39*) achieved,

may have been due to the fact that their strain was grown in the presence of tryptophane concentrations so small that the independent growth was able to escape. In our experiments the cultures were grown in the presence of optimum concentrations of tryptophane. Spontaneously occurring tryptophane-independent organisms may have been selected against, and hence could not appear when tested for on tryptophane-free medium. On the other hand, it is possible that the great stability of the tryptophane requirement of *L. arabinosus* may be due to the accumulation of mutations in *several* factors controlling several steps in the synthesis of tryptophane. Such a multiple mutant would have a very small chance of adapting through back-mutation. Once a mutation to tryptophane requirement occurred in a "wild-type" stock it is probable that additional chance mutations blocking other steps in trytophane synthesis would accumulate, owing either to the increased growth rate of the mutant—such as Roepke *et al.* (21) have found in *E. coli*—or to some other type of selection. The progressive loss of synthetic abilities and the stabilization of such heterotrophism must be involved in the evolution of organisms that are adjusting to symbiotic, parasitic, or predatory habits (*12, 18, 26*). Selective phenomena such as occur in Neurospora heterocaryons may play a role in the spread and stabilization of nutritional mutants in nature.

The pantothenic-acid requirement in *Clostridium septicum* has been completely stable in our hands. Once again, this may be due to several mutations affecting the synthesis of pantothenic acid or, since the panthenicless mutant of Neurospora is also completely stable, to the irreversible impairment or complete loss of a gene controlling pantothenic-acid synthesis.

All of the growth-factor requirements that have thus far been discussed are complete requirements. Without the growth factor no growth occurs; in its presence growth can be optimum. Another class of organism shows slow growth in the absence of the growth factor, probably due in some cases to mutant genes which are able to carry on a biosynthesis only slowly. The genes are still present, but modified. The same may be true in those strains that show a complete growth-factor deficiency under one set of cultural conditions but are able to grow under others (*31, 26*). Such adaptations may result from situations similar to the pH and temperature genic mutations in Neurospora (*3*). These mutants require growth factors only under well-defined environmental conditions. The growth-factor deficiency is not due to the loss of genes but to the modification of still self-duplicating particles.

## Conclusion

Nutritional mutants of microorganisms may adjust themselves to the deficiency of a growth factor in the environment by developing the ability to synthesize the factor. This adaptation may be brought about either by a genic mutation or by a noninherited mechanism.

In the leucineless mutants of Neurospora, adaptation is caused by a back-mutation to the wild-type condition. Every back-mutation does not result in an adaptation, however, because of a selection that occurs between the leucineless and the leucine-independent nuclei in a heterocaryon. In the presence of leucine, the leucineless nuclei have an advantage over leucine-independent nuclei. This advantage explains the greater frequency of adaptation in the presence of low concentrations of leucine.

A double leucineless mutant, in which two steps in the synthesis of leucine were blocked, was more stable than either of the single mutants. The use of this double mutant in the bioassay of leucine has the advantage that adaptations are practically nonexistent.

Among the bacteria, there is a strain of *Clostridium septicum* which requires the pyrimidine uracil for growth. This strain back-mutates to a condition where uracil is no longer required. This mutation is spontaneous in the sense that it is not induced by the absence of uracil. Uracil-independent cells also mutate to a condition of uracil-dependence. The presence of the two types of cell in an inoculum of *Cl. septicum* is probably in part responsible for the variable lag period observed in the growth of this organism in a chemically defined uracil-free medium. Similar mutations to growth-factor independence may play a role in the lag periods of other organisms and in the effect of inoculum size.

Mutations involving nutritional requirements in *E. coli* are similar to the mutations of genes in sexual organisms. The same types of biochemical deficiencies can be induced, by irradiation and by chemical means, as are induced in Neurospora (*33*). The back-mutation of these nutritional mutants is also similar. They occur in one perceptible step from a condition involving a complete loss of the ability to synthesize a growth factor to a condition indistinguishable from wild-type, and they are spontaneous in the sense that they are not induced by the absence of these factors from the medium. Mutant organisms possessing requirements for two amino acids are more stable and rarely back-mutate. The great stability of some other requirements in bacteria may be similarly due to the presence of several mutations affecting steps in the synthesis of a single growth factor.

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## DISCUSSION

RYAN: In response to Dr. Pirie's question on the efficiency with which metabolites are utilized, I would like to point out that there are some relevant data. The curve for the response of the Neurospora mutant to leucine has a slope of 2.3%. This can be compared with the leucine content of wild-type or adapted mycelium, also *ca.* 2.3%. Although the leucine content of the leucineless mold grown on limiting concentrations of leucine has not yet been directly determined, these data suggest a highly efficient utilization of leucine. Mr. Lederberg, on the other hand, has some additional relevant data, which he might wish to present in this connection.

LEDERBERG: Although a highly efficient utilization of leucine is suggested by Ryan's data, we have found that the leucine is entirely removed from the medium, in the course of the growth of the mutant long before the final weight is reached, suggesting that there is a storage or capital reserve of the amino acid under certain conditions.

In bacteria, we have some relevant data, obtained by comparing the yield of a mutant *E. coli* with the original number of cells represented in a given amount of hydrolized wild type used as a supplement for the mutant. For proline and arginine, efficiencies of about 95% were found in preliminary experiments.

ZAMENHOF: One has to bear in mind that in the study of nutrient utilization another phenomenon also may be involved and that is the resistance to the inhibitory factors produced by the cells.

It is well known that when the cells stop dividing in, let's say, a 24-hour-old broth culture, it is enough to heat the broth for half an hour at 60° to destroy the inhibitory factors, whereby upon re-inoculation the broth behaves like fresh.

This behavior of stopping the divisions long before the nutrients have been exhausted is especially striking in the case of a citrate-negative strain of *E. coli* (J. Bact. 51: 351-361, 1946). This strain mutates from citrate-negative—that is, one which cannot well utilize citrate as a sole carbon source —to citrate-positive—that is, one which can utilize it well. There is also another mutation: from the strain which is easily poisoned by the inhibitory substances (products of metabolism), and therefore stops dividing when cells reach a concentration of $10^6$/ml., to the one which is quite resistant and grows up to $10^8$ cells/ml. There is indication that these two mutations (citrate $- \rightarrow$ citrate $+$, and nonresistant $\rightarrow$ resistant) may sometimes occur independently.

RYAN: In the presence of limiting amounts of growth factor the cessation of growth of the nutritional mutants is not due to the formation of inhibitory substances. Only if new sterile growth factor is added can growth of the mutant be made to resume, and then it occurs to the extent expected on the basis of the amount of growth factor added. In such instances growth is limited *only* by the amount of growth factor present. Nor is the growth of "wild-type" mold, or of a mutant in the presence of an optimum amount of growth factor, limited by in-

hibitory substances. The medium is of such a composition that sugar is limiting. When sterile sugar is added after the cessation of growth, new growth occurs to an extent determined by the amount of sugar added. Under the conditions of our experiments, therefore, inhibitory substances, to which Dr. Zamenhof refers, do not play a role.

VAN NIEL: It should here be pointed out that the observations reported by Dr. Zamenhof may find a ready explanation along very different lines. It has long been known—and Rahn, Phelps, Rottier, E. H. Anderson, and others have made some very pertinent experimental contributions—that in most cases the growth of bacteria and other microorganisms which develop diffusely in a liquid medium is first limited by oxygen supply. This generally accounts for the enormous differences in density of population and, in later stages, in the rate of growth of cultures on the surface of agar media as contrasted with growth in liquid media. Since Neurospora is apt to grow in the form of a mat, its development in liquid cultures is not likely to be much influenced, and will usually compare favorably with that on agar plates.

The material of Dr. Ryan's paper suggests some experiments which might throw some more light on Dr. Horowitz's concept of the genesis of enzyme systems operative in biochemical syntheses. With strains deficient with respect to the synthesis of a certain vital metabolite, one might bring about the development of the proper enzyme system by incubating the culture in the presence of the known immediate precursors for this metabolite, all supplied in suboptimal concentration. Have such experiments been carried out yet?

RYAN: As yet no such experiments have been attempted, although I believe they are quite feasible. For such a study one could use a strain whose requirement for a growth factor is stable. A multiple mutant of Neurospora, containing mutant genes blocking several known reactions in the synthesis of arginine, would provide interesting material.

# THE KINETICS OF GROWTH AND MUTATION IN BACTERIA

## ARTHUR SHAPIRO

Under ordinary laboratory conditions bacteria may conveniently be studied in large numbers for many hours, while doubling in number from one to three times per hour. The obvious advantages that such rapid multiplication gives them as populations for the study of the occurrence and inheritance of mutations have been counterbalanced by specific disadvantages. These were enumerated by Shapiro, Spiegelman, and Koster (7), as follows:

"(1) The minuteness of these organisms makes many of their morphological characteristics invisible, or if visible, indistinctly so.

"(2) Those characteristics such as size, shape, capsule, staining qualities, etc., which can be distinctly observed, change so markedly with environmental conditions that genetic variations can hardly be distinguished from developmental ones. (Henrici, 1928)

"(3) Even if definite morphological criteria could be established, the necessity for isolating the individual cells observed would make the entire procedure so cumbersome that the advantages of rapid multiplication would be entirely lost."

Many of the above difficulties were overcome by employing the macroscopic properties of the cultures to characterize the cells from which they were derived. Nevertheless it was still necessary to have some way of determining how many of the mutant cells found in a culture at any time were derived from wild-type cells and how many were descendants of mutants that had arisen in previous generations. The solution to this problem involves the calculation of mutation rates.

A start in the direction of a quantitative treatment was made in 1935 by Deskowitz and Shapiro. Bunting (1) and Delbrück (2) developed their formulations along similar lines. Luria and Delbrück (6), from a different point of view, also derived a theory from which it was possible to calculate quantitative mutation rates.

In this report, the original formulation of Deskowitz and Shapiro will be extended to cover the general case of "mutation" and "reversion" of a single cell type with respect to a single characteristic while the cells are multiplying exponentially. It will be shown how mutation rates can be calculated from simple quantitative data, and an attempt will be made to interpret the observations of other workers in terms of the theory.

Our attack on this problem began about thirteen years ago, during some experiments on the factors influencing variation in colony form of *Salmonella aertrycke*, when it was noted that certain "unstable" variant strains behaved in a strikingly reproducible manner. Repeated serial plating out of well-isolated colonies consistently gave a constant percentage of colonies different in appearance from the parent type. A number of such "unstable" variant strains were isolated and subsequently described by Deskowitz (3).

One of these, M.T. 2 CR, was found by Deskowitz and Shapiro (4) to be especially suitable for quantitative study because the typical colony—

## DIAGRAM of BEHAVIOR of M.T. 2 CR CULTURES

A. On infusion agar.
B. In infusion broth.
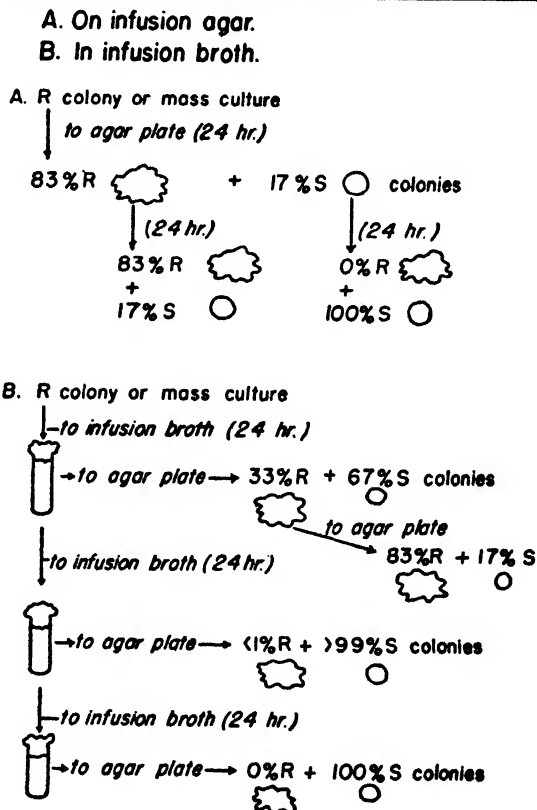


FIG. 1.

large, flat, and rough—gave on plating out approximately 83% colonies of the original rough and 17% small, smooth, dome-shaped colonies. The smooth colonies were easily distinguishable from the original rough, and always yielded 100%

smooth colonies in repeated passage. When a rough colony was inoculated into broth, a higher percentage of smooth colonies was found on plating out after 18 hours and a pure smooth culture after 2 or 3 serial passages in broth. (Fig. 1)

These observations suggested that the appearance of smooth colonies on plates streaked from rough colonies might be due to the occurrence of spontaneous mutations from rough to smooth cells, in the developing rough cultures. In order to think clearly about the quantitative aspects of this problem, it was necessary to use the concept of a smooth *cell* as one which gives rise to a smooth colony and a rough *cell* as one which gives rise to a rough colony on standard agar test plates. This concept was rigorously analyzed in a subsequent paper by Shapiro, Spiegelman, and Koster (7) and shown to be valid.

In interpreting the behavior of the cultures in broth and on agar, it became necessary to determine whether the difference was due to a change in growth rate of $R$ or $S$ cells in broth as compared with agar, a change in mutation rate, or both.

To answer these questions, an analysis of the exponential phase of the growth of these cultures was proposed. Since the original article by Deskowitz and Shapiro (4) was very much abbreviated, and has escaped the attention of many of those working with the genetics of microorganisms, it will be presented here in some detail.

Let n equal the total number of cells per unit volume of culture; r the number of $R$ cells per unit volume of culture; s the number of $S$ cells per unit volume of culture; and t the time of growth.

We assume that for a particular medium, during the exponential phase of growth, the rate of division of each type of cell is constant. Then, since $R$ cells do not arise from $S$ cells, we may express the rate of increase of $R$ cells in the culture as:

$$\frac{dr}{dt} = cr \tag{1}$$

where c is a constant.

$S$ cells may arise from either $R$ cells or $S$ cells. Using assumptions analogous to those for the rate of increase of $R$ cells, we find that the rate of formation of $S$ cells from $S$ cells at any time is proportional to their number in unit volume of culture, while the rate of formation of $S$ cells from $R$ cells is proportional to the number of $R$ cells in unit volume of culture. The rate of increase of $S$ cells in the culture, then, may be written as:

$$\frac{ds}{dt} = as + br \tag{2}$$

where a and b are constants—a the exponential-growth constant for $S$ cells, and b the mutation-rate constant for the occurrence of $S$ cells as

mutations of $R$ cells. The constant b is measured in mutations per bacterium per hour. It is interesting to note that the fraction of the total number of cells arising from $R$ cells which are $S$ is expressed by the constant $b/(b+c)$, which is independent of the time units used in measuring the constants.[1]

When equations (1) and (2) are integrated and the integration constants determined by setting $r = r_0$, $s = s_0$, when $t = 0$, we get:

$$r = r_0 e^{ct} \tag{3a}$$

$$s = \left(s_0 - \frac{br_0}{c-a}\right)e^{(a-c)t} + \frac{b}{c-a} \tag{3b}$$

$$\frac{s}{r} = \left(\frac{s_0}{r_0} - \frac{b}{c-a}\right)e^{(a-c)t} + \frac{b}{c-a} \tag{3c}$$

provided $c \neq a$.

When $c = a$, substituting in (1) and (2) and integrating gives for (3b) and (3c):

$$s = (s_0 + br_0 t)e^{ct} \tag{3b'}$$

$$\frac{s}{r} = \frac{s_0}{r_0} + bt. \tag{3c'}$$

Now if $c > a$, equation (3c) gives:

$$\lim_{t \to \infty} \frac{s}{r} = \frac{b}{c-a}. \tag{4a}$$

When $c \leqq a$, equations (3c) and (3c') give:

$$\lim_{t \to \infty} \frac{s}{r} = \infty. \tag{4b}$$

The differential equations (1) and (2) lead, then, to the conclusion that exponentially growing cultures of M.T. 2 CR approach a limiting ratio of mutants to wild-type cells if allowed to grow long enough, provided only that c, the constant expressing the rate of formation of wild-type cells from wild-type cells, is greater than a, the constant expressing the rate of formation of mutant-type cells from mutant-type cells. This is true regardless of the value of b, the constant expressing the rate of formation of mutants from wild-type cells, although the magnitude of the ratio of mutants to wild type in a culture at equilibrium in exponential growth is given by the quantity $b/(c-a)$.

On the other hand, if $a \geqq c$, the culture approaches the composition of a pure culture of mutants. Only the rate at which this happens depends on the magnitude of b. It would be pre-

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[1] Delbrück (2) has set up and solved equations equivalent to (1) and (2) as his *case a*. His solution applies to the special case of $s_0/r_0 = 0$, and his discussion does not consider the possibility of $a \geqq c$.

dicted, then, that for mass cultures growing on agar, $c > a$, for mass cultures growing in broth $c \leq a$. An experimental test of these predictions could be performed if the numerical values of the constants a, b, c could be measured separately in broth and on agar.

As has already been noted, on agar, exponentially growing mixtures of $R$ and $S$ organisms approach a limiting population where

$$\lim_{t \to \infty} \frac{s}{r} = \frac{b}{c-a}.$$

If an agar plate is uniformly inoculated (by spraying a suspension) with such a culture,

$$\frac{s_0}{r_0} = \lim_{t \to \infty} \frac{s}{r} = \frac{b}{c-a}.$$

If we substitute this in equations (3a) and (3b) and add, we get:

$$r + s = (r_0 + s_0)e^{ct} = n = n_0 e^{ct}. \tag{5a}$$

Then

$$c = \frac{1}{t} \ln \frac{n}{n_0} = \frac{2.30}{t} \log \frac{n}{n_0}.$$

The value of c on agar can thus be calculated by measuring the slope of a plot of log n against t for an $R$ culture inoculated with a suspension that has already reached its equilibrium composition. The value of a for growth on agar can be obtained from a similar plot for a pure $S$ culture.

The difference between the directly measured values of a and c can be used to calculate b from the equilibrium value of $b/(c-a)$, but this would involve the error due to using as a factor a small difference between large quantities. A better way to calculate $(c-a)$ for growth on agar is to rewrite equation (3c) as:

$$\left(\frac{s}{r}\right) - \left(\frac{s}{r}\right)_\infty = \left[\left(\frac{s}{r}\right)_0 - \left(\frac{s}{r}\right)_\infty\right]e^{(a-c)t}$$

$$a - c = \frac{1}{t} \ln \frac{(s/r) - (s/r)_\infty}{(s/r)_0 - (s/r)_\infty}$$

$$= \frac{2.30}{t} \log \frac{(s/r) - (s/r)_\infty}{(s/r)_0 - (s/r)_\infty}. \tag{3d}$$

Thus $(a-c)$ may be directly determined from the slope of a plot of log $[(s/r) - (s/r)_\infty]$ against time for mixture of $S$ and $R$ organisms growing exponentially on agar and approaching equilibrium.

In broth cultures, the slope of the logarithmic plot of the growth curve was found to be independent of the percentage of smooth cells. Adding equations (1) and (2) gives:

$$\frac{dr}{dt} + \frac{ds}{dt} = \frac{dn}{dt} = as + (b+c)r. \tag{6}$$

Thus dn/dt can be independent of s/r only if
$$a = b + c. \tag{6a}$$
For this condition, equation (6) becomes:

$$\frac{dn}{dt} = a(r+s) = an$$

whence $n = n_0 e^{at}$

$$a = \frac{1}{t} \ln \frac{n}{n_0} = \frac{2.30}{t} \log \frac{n}{n_0}$$

and a may be measured directly on either a rough or a smooth culture.

To evaluate b in broth, substitute $a = b + c$ in (3c).

$$\frac{s}{r} = \left(\frac{s_0}{r_0} + 1\right)e^{bt} - 1 \tag{5b}$$

$$b = \frac{1}{t} \ln \left(\frac{1+s/r}{1+s_0/r_0}\right)$$

$$= \frac{2.30}{t} \log \left(\frac{1+s/r}{1+s_0/r_0}\right). \tag{6b}$$

Thus b may be measured from the slope of a plot of log $(1+s/r)$ against t for a culture inoculated with a suspension containing a high percentage of $R$ cells, and c may be determined from $a = b + c$.

The observed values of the constants measured in the manner indicated above are given in Table 1, from which it is clear that the difference in the behavior of the culture in broth and on agar was due to a change in the value of a, the growth constant of the pure $S$ culture. The mutation rate b was constant to well within the limits of experimental error.

The finding of a constant mutation rate as a consequence of the application of equations (1) and (2) to quantitative data on M.T. 2 CR cultures growing exponentially in broth and on agar was considered something of a triumph in 1935. However, when Bunting (1) reported the data on the behavior of pigmented variants of *Serratia marcescens* #274 which she has discussed in detail at this Symposium, a more general analysis was found to be necessary.

For complete generality, we shall consider two bacterial "types," X and Y, so interrelated genetically that in a large number of cell divisions each gives rise to a definite fixed proportion of cells of each of both types.

We shall assume that in a time interval, dt, during exponential growth, x cells of X type will give rise to axdt cells of X type and mxdt cells of

TABLE 1.* OBSERVED VALUES OF GROWTH AND MUTATION-RATE CONSTANTS OF SALMONELLA AERTRYCKE
M.T. 2 CR, IN BACTERIA PER BACTERIUM PER HOUR

| Conditions of Growth | Growth-Rate Constant for $S$ cells a | Mutation-Rate Constant for $R$ cells b | Growth-Rate Constant for $R$ cells c | $\dfrac{b}{b+c}$ |
|---|---|---|---|---|
| $S$ in broth | 1.26±.02 P.E. | | | |
| $R$ in broth | 1.30±.02 P.E. | 0.059±.001 P.E. | 1.24±.02 P.E. | .045 |
| $S$ on agar | 1.04±.02 P.E. | | | |
| $R$ on agar | | 0.061±.001 P.E. | 1.22±.02 P.E. | .048 |

* From Deskowitz and Shapiro, 1935.

Y type. During the same interval, y cells of Y type give rise to cydt cells of Y type and bydt cells of X type. If a, b, c, m, are constants greater than or equal to zero, and x and y represent the number of X and Y cells respectively present in the cultures at time t, we may write for the rates of increase of X and Y cells in the culture:

$$\frac{dx}{dt} = ax + by \qquad (7a)$$

$$\frac{dy}{dt} = mx + cy. \qquad (7b)$$

Integrating (7a) and (7b) and determining the integration constants by setting $x = x_0$, $y = y_0$, when $t = 0$, we get:

$$x = \frac{by_0 - (q-a)x_0}{p-q} e^{pt} - \frac{by_0 - (p-a)x_0}{p-q} e^{qt} \qquad (8a)$$

$$y = \frac{mx_0 - (q-c)y_0}{p-q} e^{pt} - \frac{mx_0 - (p-c)y_0}{p-q} e^{qt} \qquad (8b)$$

$$\frac{y}{x} = \frac{m - (q-c)\dfrac{y_0}{x_0} - \left[ m - (p-c)\dfrac{y_0}{x_0} \right] e^{(q-p)t}}{b\dfrac{y_0}{x_0} - (q-a) - \left[ b\dfrac{y_0}{x_0} - (p-a) \right] e^{(q-p)t}} \qquad (8c)$$

where

$$p = \tfrac{1}{2}(a + c + \sqrt{(a-c)^2 + 4bm}) \qquad (9a)$$

$$q = \tfrac{1}{2}(a + c - \sqrt{(a-c)^2 + 4bm}). \qquad (9b)$$

Equations (8a, b, c) are valid as long as $p - q \neq 0$; that is unless $(a-c)^2 = 0 = 4bm$.

If $b = m = 0$, $bm = 0$, but X and Y are unrelated types.

If b or $m = 0$, the case is that covered by equations (1) and (2) and already fully discussed.

Now $p - q = \sqrt{(a-c)^2 + 4bm} > 0$. Therefore the exponential terms in equation (8c) approach zero as a limit when t approaches infinity, and:

$$\lim_{t \to \infty} \frac{y}{x} = \frac{m - (q-c)\dfrac{y_0}{x_0}}{b\dfrac{y_0}{x_0} - (q-a)}.$$

This seems to indicate that the equilibrium composition of the culture depends on the initial composition $y_0/x_0$. But if we note that $(q-a)(q-c) = bm$, we get:

$$\lim_{t \to \infty} \frac{y}{x} = \frac{c-q}{b} = \frac{c-a}{2b} + \frac{m}{b}\sqrt{1 + \left(\frac{a-c}{2m}\right)^2} \qquad (10)$$

which is independent of $y_0/x_0$ and contains only $(c-a)$, the selection rate; m, the mutation rate from X to Y; and b, the mutation rate from Y to X.

The constants a, b, c, m for the general case where $bm \neq 0$, may be evaluated by procedures similar to those used for the case where $m = 0$.

It is convenient, first, to evaluate the quantities $\theta_\infty$, A, p, q, where $\theta_\infty = \lim\limits_{t \to \infty} x/y$ and A is the coefficient of $e^{pt}$ in equation (8a).

If $x_0/y_0$ is chosen equal to $\theta_\infty$, then:

$$p = \frac{1}{t} \ln \frac{n}{n_0} = \frac{2.30}{t} \log \frac{n}{n_0}. \qquad (11a)$$

If we eliminate $e^{qt}$ from equations (8a) and (8b) it can be shown that:

$$A = \frac{x_0 y - y_0 x}{(y - y_0)e^{pt} - \theta_\infty(x - x_0)}. \qquad (11b)$$

Then A can be evaluated by first determining $\theta_\infty$ and p and then measuring n and x/y at various times t. From these, x and y can be calculated, and, by substituting in equation (11b), A may be determined.

It can easily be shown that:

$$q = \frac{1}{t} \ln \frac{x - Ae^{pt}}{x_0 - A} = \frac{2.30}{t} \log \frac{x - Ae^{pt}}{x_0 - A} \qquad (11c)$$

so that q may be determined from the slope of a plot of log $(x - Ae^{pt})$ against t made with the data used in computing A.

Although there are many steps involved in the calculations, the parameters $\theta_\infty$, p, q, and A are not too difficult to determine experimentally. Then, with the values of $x_0$ and $y_0$ used for computing A, the following formulas give the values of a, b, c, m.

1.  $b = \dfrac{(x_0 - A)(p - q)}{\theta_\infty x_0 - y_0}$

2.  $a = p - p\theta_\infty$           (11d)

3.  $c = p + q - a$

4.  $m = \theta_\infty(a - q)$.

The theoretical development given in equations (7a) to (11d) provides a means for investigating the quantitative changes in growth-rate constants and mutation-rate constants for exponentially growing cultures where both forward and backward mutation rates are finite. Unfortunately, no data are available that require the complete solution with all four constants independent of each other.

It is interesting, however, to apply our solution to the data given by Bunting (*1*) for *Serratia marcescens* #274. Since both dark-red and bright-pink colonies occur in subcultures from colonies of either type, we may express the mutation and growth process as:

$$\overline{P} \rightarrow \overline{P} + \overline{R}$$
$$\overline{R} \rightarrow \overline{P} + \overline{R}$$

where $\overline{P}$ and $\overline{R}$ refer to bright-pink and dark-red cells respectively. Thus a, b, c, and m of equations (1) and (2) are finite. But since it was observed that the generation time of the culture was independent of the percentage of $\overline{P}$ or $\overline{R}$ cells, the four constants are not independent of one another.

Adding (7a) and (7b) gives:

$$\frac{dx}{dt} + \frac{dy}{dt} = (a + m)x + (b + c)y. \qquad (12a)$$

For *S. marcescens* #274, if we let x = P be the number of pink cells and y = R be the number of red cells, we may write:

$$\frac{dR}{dt} + \frac{dP}{dt} = k(R + P). \qquad (12b)$$

This can be true only when

$$a + m = b + c = k. \qquad (12c)$$

For these conditions we get from equation (9), since $a - c = b - m$:

$$p = \tfrac{1}{2}(c + a - \sqrt{(b - m)^2 + 4bm})$$
$$= \tfrac{1}{2}(a + c + b + m) = k. \qquad (13a)$$

Since p + q = c + a,

$$q = c + a - k = c + a - a - m = c - m = a - b \quad (13b)$$

Then equations (3a) and (3b) become:

$$P = \frac{b}{b + m}(R_0 + P_0)e^{kt} + \frac{mP_0 - bR_0}{m + b}e^{(a-b)t} \quad (14a)$$

$$R = \frac{m}{b + m}(R_0 + P_0)e^{kt} - \frac{mP_0 - bR_0}{m + b}e^{(a-b)t} \quad (14b)$$

whence:

$$R + P = n = (R_0 + P_0)e^{kt} = n_0 e^{kt}. \qquad (14c)$$

Since for these conditions (R + P) is so simple in form we shall use $\psi = (R \times 100)/(R + P)$ as a measure of the composition of the culture instead of R/P. From equations (14a, b, c) we get:

$$\frac{R \times 100}{R + P} = \psi = \frac{m \times 100}{m + b} - \left[ \frac{m}{m + b} \cdot \frac{100P_0}{R_0 + P_0} \right.$$
$$\left. - \frac{b}{m + b} \cdot \frac{100R_0}{R_0 + P_0} \right] e^{-(m+b)t} \qquad (14d)$$

Here

$$\lim_{t \to \infty} \frac{R \times 100}{R + P} = \lim_{t \to \infty} \psi = \psi_\infty = \frac{m \times 100}{m + b} \cdot \quad (14e)$$

Then (14d) may be written as:

$$\psi - \psi_\infty = (\psi_0 - \psi_\infty)e^{-(m+b)t}. \qquad (14f)$$

The constants for this special case are easily determined from the data of Bunting (*1*).

From equation (14c)

$$k = \frac{1}{t} \ln \frac{n}{n_0} = \frac{2.30}{t} \log \frac{n}{n_0} \qquad (15a)$$

or, since the culture doubles every 64 minutes,

$$k = \frac{2.30}{64 \text{ minutes}} \log 2 = \frac{2.30 \times 0.301}{0.045 \text{ days}}$$
$$= 15.5/\text{day} = 0.69/\text{div. cycle}.$$

From equation (14f)

$$-(m + b) = \frac{1}{t} \ln \frac{\psi_\infty - \psi}{\psi_\infty - \psi_0} = \frac{2.30}{t} \log \frac{\psi_\infty - \psi}{\psi_\infty - \psi_0} \cdot (15b)$$

Fig. 2 shows a plot of log $(\psi_\infty - \psi)$ against time in days. Its slope, calculated by least squares, is −0.0747 per day, which gives

$$m+b=2.30\times0.0747=0.172/\text{day}$$
$$=7.7\times10^{-3}/\text{div. cycle.}$$

From equation (14e) and Bunting's data:

$$\psi_\infty=\frac{m\times100}{m+b}=97 \qquad \frac{m}{m+b}=0.97.$$

The values of growth and mutation-rate constants for *Serratia marcescens* #274 calculated from Bunting's data (*1*) are shown in Table 2.
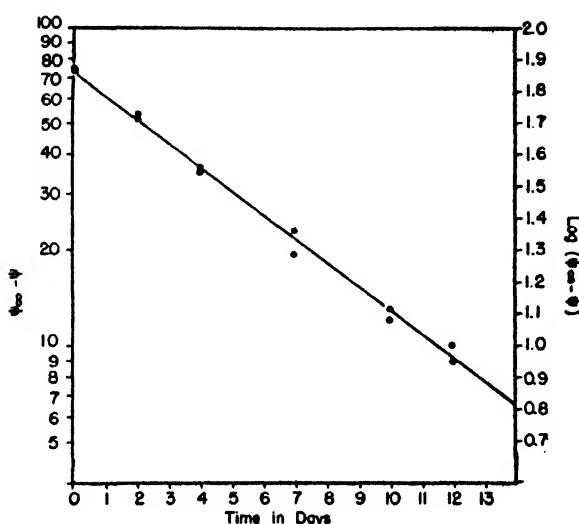


Fig. 2. A graph of the logarithm of $(\psi_\infty-\psi)$ as a function of time for *Serratia marcescens* #274. The points are calculated from Bunting's (*1*) data, Table 1, columns 5 and 6. The line is drawn to the least squares solution.

Bunting (*1*), in her own mathematical analysis of this data, derived a formula from an analysis of equilibrium conditions and the assumption that the percentage of cells of each type giving rise to

Table 2. Values of Growth and Mutation-Rate Constants for Serratia marcescens #274 Calculated from Bunting's Data (*1*)

| Constant | Number per Bacterium per Day | Number per Bacterium per Division Cycle |
|---|---|---|
| m | 0.167 | $7.5\times10^{-3}$ |
| b | 0.0052 | $2.3\times10^{-4}$ |
| c | 15.5 | $6.9\times10^{-1}$ |
| a | 15.3 | $6.8\times10^{-1}$ |

mutants in any finite time interval is a constant. Rewriting her equation in the terminology of this paper, simplifying the algebraic form, and reducing it to an expression analogous to equation (14f) gives:

$$\psi-\psi_\infty=\psi_0-\psi_\infty-\frac{\psi_0-\psi_\infty}{100-\psi_\infty}R_p\times100 \qquad (16)$$

where $R_p$ is the fraction of dark-red cells giving rise to bright-pink cells in the time interval t. Comparison with equation (14f) shows that equation (16) can be true only if:

$$R_p=\frac{100-\psi_\infty}{100}(1-e^{-(m+b)t}). \qquad (17)$$

A calculation for $t=2$ days, the time interval used by Bunting (*1*), but using the constants derived from the whole set of data, gives $R_p=0.0087/2$ days, a value in good agreement with the value of 0.0099 which she calculated from data on a single 2-day interval.

The methods and analysis given above for the measurement of mutation rates are straightforward and, theoretically at least, simple. They have thus far been applied only to cultures with mutation rates from $4\times10^{-2}$ to $2\times10^{-4}$ mutations per bacterium per division cycle.

Luria and Delbrück (*6*) have stated: "It is clear, however, that this method is applicable only in cases of mutation rates of at least $10^{-4}$ per bacterium per division cycle." And Delbrück (*2*) has said: "To sum up this matter, we may say that the determination of mutation rate from the number of mutants is necessarily a statistically inefficient procedure." The analysis which leads to their statements, however, rests on the fact that under certain conditions similarly inoculated cultures will differ very markedly from each other in the number of mutants present after a given time of growth. Luria and Delbrück (*6*) have themselves precisely formulated the conditions under which these large deviations may be expected to occur, as follows: " ... *the initial number of bacteria in a culture must be so small that the number of mutations which occur during the first division cycle of the bacteria is a small number.* This will always be true, however great the mutation rate if one studies cultures containing initially a small number of organisms."

On the other hand, it is also true that these large deviations may be made negligibly small, even for very small mutation rates, by studying cultures containing, initially, a large number of organisms. For example, Luria and Delbrück's sensitive strain of *B. coli* had a calculated mutation rate of about $2\times10^{-8}$ per bacterium per division cycle, and a maximum population of about $4\times10^{10}$ bacteria per 10 cc. If, instead of studying 10-cc. cultures inoculated with 50 to 500 cc. of bacteria, they had studied 1000-cc. cultures inoculated with $4\times10^{10}$ bacteria, the number of mutants present initially would have been about $7.5\times10^2$ and the number of mutations in the first division cycle about $2\times10^2$. It is easy to calculate that the cul-

ture would take a little more than two hours to reach its maximum population and that serial passage every two hours would keep it growing exponentially.

Let us see what we can learn about this culture by applying our theory. Let y equal the number of sensitive bacteria in 10 cc., x the number of resistant bacteria in 10 cc. Equation (8c) and its special forms (3c), (3c'), and (14f) are all expressions for the *ratio* of the number of mutants to either the number of wild-type cells or the total number of cells per unit volume of culture at any time, and they are all independent of the actual *number* of cells of either type. They may therefore be applied to exponentially growing cultures regardless of the fact that these are diluted at regular intervals with fresh culture medium.

For their cultures, Luria and Delbrück found no measurable difference between the growth rates of the sensitive and resistant cultures, and no measurable reversion of resistant to sensitive bacteria. We may write, then, for their cultures:

$$a+m=b+c\pm\delta$$
$$m=\epsilon \tag{18}$$

where $\epsilon$ and $\delta$ are either zero or quantities too small to be detected by their methods. For the moment let us consider that they are both zero. Then equation (18) is equivalent to the conditions for the growth of M.T. 2 CR in broth, which gives the equivalent of (5b) and (6b), so that we may write for the mutation rate b:

$$b=\frac{1}{t}\ln\frac{1+\dfrac{x}{y}}{1+\dfrac{x_0}{y_0}}=\frac{2.30}{t}\log\frac{1+\dfrac{x}{y}}{1+\dfrac{x_0}{y_0}} \tag{18a}$$

and the time required to reach any particular value of x/y would be:

$$t=\frac{1}{b}\ln\frac{1+\dfrac{x}{y}}{1+\dfrac{x_0}{y_0}}=\frac{2.30}{b}\log\frac{1+\dfrac{x}{y}}{1+\dfrac{x_0}{y_0}}\cdot \tag{18b}$$

The initial value of x/y would be of the order of $2\times10^{-8}$, and the mutation rate about $6\times10^{-8}$ per bacterium per hour. Then the time taken for x/y to increase one-hundredfold would be:

$$t_{100}=1.7\times10^7\ln\frac{1+\left(100\dfrac{x_0}{y_0}\right)}{1+\dfrac{x_0}{y_0}}$$

or, approximately, if $x_0/y_0$ is less than $10^{-8}$:

$$t_{100}=1.7\times10^7\times100\frac{x_0}{y_0}=1.7\times10^9\frac{x_0}{y_0}\text{ hours.}$$

Then, for example, if we start with 10 cc. of ordinary sensitive culture at full growth, $t_{100}=2\times10^{-8}\times1.7\times10^9$ hours $=34$ hours. This is an experiment which can easily be carried out to test the hypotheses. If it does not check, other assumptions must be made about $\delta$ and $\epsilon$, and similar crucial experiments set up and tried. In any case, it is clear that small differences in selection rate and small values of mutation rate can be detected by an analysis of the behavior x/y as a function of time, even when mutation rates are in the neighborhood of $10^{-8}$ per bacterium per division cycle.

## CONCLUSION

An analysis of the processes of mutation and multiplication in exponentially growing cultures of bacteria has led to the simple assumptions of constant growth and mutation rates for any particular culture under specified conditions. The differential equations expressing these assumptions have given solutions which agree quantitatively with whatever data are available and suggest new experiments to test conclusions reached by other analyses.

For many cases, the expression for the frequency with which mutants are found in the population as a function of time of growth leads to an equilibrium population in which both wild type and mutant occur in a fixed ratio. For other cases, the culture can be at equilibrium only as a pure culture of the mutant. For all of these cases precise methods of computing the values of the specific growth and mutation-rate constants are available.

It is to be hoped that future investigators will use, correct, and improve on the mathematical and technical tools described and applied in this paper.

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## DISCUSSION

ZAMENHOF: It seems that there is no definition of mutation rate in microorganisms to which everybody would agree. The definition referring to unit of time (for instance, number of mutants per number of mother cells per day) is not correct if one agrees with the very probable theory that the mutations in microorganisms can occur only during cell divisions; in this latter case the definition "per one cell division" or "per generation" would be correct. It seems, therefore, that before one proceeds with mathematical formulation of mutation rate one should first solve this basic problem: are mutations possible without cell divisions? Incidentally, the solution of this problem is of great importance also for the problem of whether mutations of viruses *in vitro* are possible or not.

# EXPERIMENTAL CONTROL OF THE CONCENTRATION OF CYTOPLASMIC GENETIC FACTORS IN PARAMECIUM[1]

## T. M. SONNEBORN[2]

One method of controlling the concentration of a cytoplasmic factor is to control the amount of cytoplasm transferred from one conjugant to its mate during conjugation. This method has been discussed in previous papers (*11, 12*); it will be reexamined here in Section III in the light of newer knowledge. Two new methods of controlling the concentration of cytoplasmic factors have recently been discovered. Preer (*7*) has been able to accomplish this by varying the rate of reproduction of the animals; his work is reviewed in Section II. The third method is set forth for the first time in Section IV. It consists of destruction of cytoplasmic factors by exposure to high temperature, the amount destroyed and the amount remaining being controlled by the duration of exposure. These three methods have led and should continue to lead to results that bear on questions of general interest, as pointed out throughout this paper and as discussed particularly in Section V. To provide the necessary background, the main facts concerning the determination and inheritance of the characters under examination are briefly reviewed in Section I.

## I. Determination and Inheritance of the "Killer" Character

The pair of alternative characters in *Paramecium aurelia* with which this study is concerned are designated "killer" and "sensitive." Stocks that make the fluid in which they live poisonous for other stocks are known as killers. Stocks that are killed by such fluid are known as sensitives. Killer stocks have been reported (*9, 10*) in varieties 2 and 4 of *Paramecium aurelia*. The killer that has been most fully studied (*10, 11, 12, 13*) is stock 51 of variety 4 of *P. aurelia*. When sensitive stocks of variety 4 are exposed to the poisonous fluid from cultures of the killer stock 51, they develop the morphological changes illustrated diagrammatically in Fig. 1. A slight hump appears after several hours on the aboral surface near the hind end of the body. This hump enlarges while the anterior part of the body gradually wastes away and the posterior part of the body is pushed into the humped region.

The animals then become smaller and spherical and finally die. Sensitives can be mated to killers without any evidence of injury, if mating begins soon after the two kinds of paramecia are brought together, if the conjugant pairs are removed to fresh culture fluid soon after they unite, and if the two members of each conjugant pair are put into separate culture dishes soon after conjugation has been completed.

The killer phenotype is manifested only when there is present in the cytoplasm a factor known as
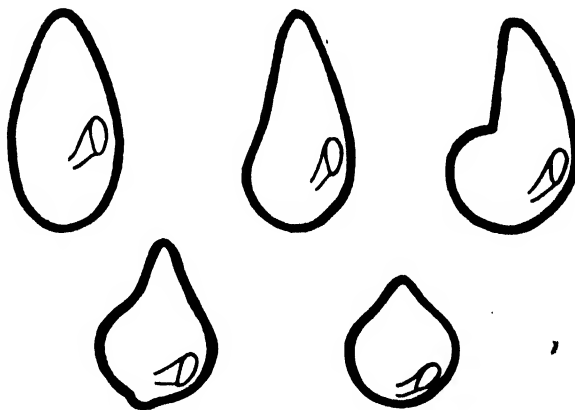


FIG. 1. The sequence of morphological changes preceding death when sensitive animals of *P. aurelia* are exposed to the action of killer stock 51 of variety 4.

"kappa." In the absence of kappa the phenotype is invariably sensitive. The presence of kappa in the cytoplasm is related in a peculiar way to the genetic constitution. Clones of killers always have in the nucleus a dominant gene $K$, either in homozygous or heterozygous condition. If the allele $k$ is substituted for gene $K$, the kappa which is initially present in the cytoplasm soons disappears; the resulting individuals transform into sensitives and reproduce thereafter true to this character. Once kappa has disappeared from the cytoplasm it cannot be brought back by restoring gene $K$. Sensitive stocks, therefore, may have either allele, $K$ or $k$, for kappa is not initially producible by any known gene.

The relations between $K$ and kappa are further illustrated by Fig. 2. When a cross is made between killers and sensitives both of which are homozygous for $K$, each member of a conjugant pair usually

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gives rise to a clone of the same character as the parent from which it derives the bulk of its cytoplasm. Normally, there is no cytoplasmic exchange between the mates; hence, usually the killer conjugant produces a killer clone and the sensitive conjugant produces a sensitive clone. Under certain conditions, however, as shown in the lower part of Fig. 2, exchange of cytoplasm does take place between the mates. When this happens, both mates produce killer clones. This illustrates what has been designated elsewhere (*12*) as the primer relation between kappa and *K*. When cytoplasm containing
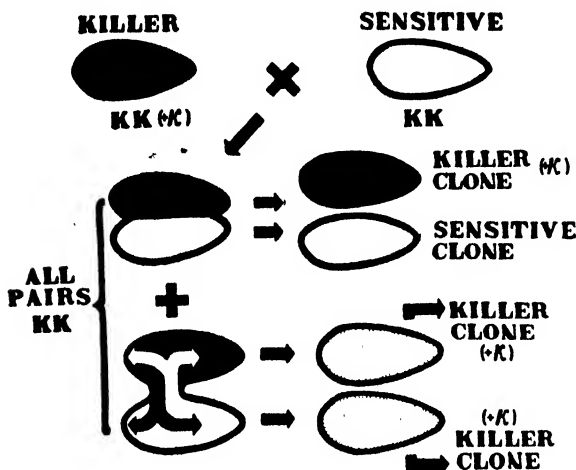


Fig. 2. Inheritance of killer and sensitive in crosses of *KK* killers (containing kappa) by *KK* sensitives (lacking kappa). The middle row shows inheritance when there is effectively no transfer of cytoplasm between mates; the bottom row shows inheritance when much cytoplasm is exchanged between mates.

kappa is introduced into a sensitive animal containing the gene *K*, kappa is maintained and multiplied thereafter in all subsequent vegetative and sexual reproduction. Kappa thus acts like a primer in a pump. A little kappa is put in and more kappa comes out. The function of the gene K seems to be to control the maintenance and increase of kappa when some is already present, although it cannot start the production of kappa when none is initially present.

## II. CONTROL OF THE CONCENTRATION OF KAPPA BY CONTROLLING THE RATE OF FISSION OF THE PARAMECIA

This section is devoted to an account of killers in variety 2 of *P. aurelia*. The "varieties" of *P. aurelia* are sexually isolated from each other and may therefore be considered as different species (*14*). Killer stocks in variety 2 differ from each other and from the variety 4 killer stock 51 (discussed in Section I) in the types of effects produced on sensitive animals before the sensitives are killed. Pre-

liminary studies (unpublished) performed 7 years ago on the inheritance of these killer characters showed them to follow the scheme indicated in the middle of Fig. 2. With this information alone, it was impossible to decide whether the characters were cytoplasmically inherited or whether there was regularly no exchange of nuclei between mates during conjugation. In view of present knowledge of the inheritance of the killer character in variety 4, however, there can be little doubt that the former alternative is the correct one. The killer characters in variety 2 must be determined by cytoplasmic factors comparable to kappa in variety 4. They also may be designated by the term kappa, without implying necessary identity between the kappas in different stocks and varieties.

Early in the course of an attempt to reinvestigate the mechanism of determination and inheritance of the killer characters in variety 2, Preer (*7*) made a remarkable observation, which provided a means of discovering the concentration and rate of increase of cytoplasmic factors. He observed that clones of these stocks remained pure killers only when they were grown slowly by controlling the amount of food supplied. When clones of these same killer stocks were fed more abundantly and reproduced more rapidly, some sensitive animals appeared in the cultures. Preer found further that, if the killers reproduced rapidly for a relatively short period the resulting sensitive animals could be made to revert to killers simply by cutting down the food supply so as to slow up or suppress reproduction. If the killers reproduced rapidly for a longer period of time, however, then not all of the sensitives could be made to revert to killers by retarding or suppressing their reproduction. Some remained pure sensitives and could never thereafter be made to revert to killers during either vegetative or sexual reproduction. Further, the percentage of such irreversibly pure sensitives increased as the period of rapid fission was prolonged.

Preer isolated a single killer animal (from stock G, variety 2) and allowed it to multiply at a rate of about 3.4 fissions per day. From this single ancestor, 192 individuals were separately cultivated as daily isolation lines of descent, a single individual from each of the 192 lines being reisolated every day. After various intervals during the period of rapid reproduction, the animals left over in each line after the daily isolations had been made were allowed to grow very slowly or not at all for a considerable period and then tested to find out how many of the 192 lines at this stage consisted of irreversibly pure sensitives. The percentage of lines that were pure sensitives increased from 1% after 12 rapid fissions to 99% after 36 rapid fissions.

As the pure sensitives must have lost kappa, the percentage of animals that lost kappa increased with time during rapid reproduction. This indicates that the paramecia multiplied more rapidly than kappa.

As a consequence, the average concentration of kappa in the animals decreased, and an increasing proportion of the animals came to lack it entirely.

Certain important calculations can be made if it is assumed (1) that at the time of fission the particles of kappa in an animal are randomly distributed to the two daughter animals, and (2) that if an animal has one particle of kappa and its reproduction is stopped there will eventually be produced within the animal enough kappa to make it a killer. As will appear below, both asumptions have been verified experimentally. From these assumptions, it follows that the irreversibly pure sensitives are those animals which by chance have received not a single particle of kappa. The frequency with which such animals arise would be $e^{-m}$, where m is the mean number of kappa particles per animal at the time a test is made, e is the base of the natural logarithms, and $e^{-m}$ is the first term of the Poisson series. (The Poisson distribution is not strictly applicable to the present case because the particles being distributed are also increasing in number during the sampling process. Preer has shown, however, that the error involved is small; hence, the uncorrected calculations based on $e^{-m}$ will serve present purposes.) The mean number of particles of kappa per animal, calculated from the percentage of irreversibly sensitive animals, is 1.4 after 16 rapid fissions, and 0.3 after 21 rapid fissions. As each animal produced 32 progeny in this period, the 1.4 particles per animal after the 16th fission increased to 32 × 0.3, or 9.6, particles after the 21st fission. This represents a 6.9-fold increase in kappa, or $(2)^{2.8}$—that is, the equivalent of 2.8 doublings. Thus, the animals underwent 5 doublings while kappa underwent 2.8 doublings, or kappa underwent the equivalent of 0.56 doubling for each fission or doubling of the animals.

With this information, the number of particles in the original killer animal may be computed. In 16 fissions, this animal produced $(2)^{16}$ or 65,536 progeny, with a mean of 1.4 particles of kappa per animal. The total number of particles of kappa after 16 fissions is thus 1.4 × 65,536, or 91,750. In 16 fissions there were at the same time 16 × 0.56 or approximately 9 doublings of each of the particles of kappa present in the original killer; in other words, each original particle of kappa produced $(2)^9$, or 512, of the 91,750 particles present after 16 fissions. Dividing 91,750 by 512 gives about 180 as the number of particles of kappa present in the original killer animal; and, allowing for the correction mentioned above, the calculation comes to 256 instead of 180. This number is, of course, subject to minor errors and is to be understood only as indicating order of magnitude.

Certain of the assumptions made in arriving at these estimates of particle number and rate of increase of kappa may now be tested. The first to be tested is the assumption that the particles of kappa

are distributed at random to the two daughter animals in the process of fission. The test of this assumption is to see whether one can, by using the Poisson series in the way already illustrated, predict the percentage of irreversibly sensitive animals at a later stage, such as the 26th fission, which is 5 fissions later than the last one previously considered. Since the Poisson series would be expected to apply only if there is random distribution of the particles, the applicability of the theorem constitutes a test of the random distribution of kappa. One would again expect a 6.9-fold increase in the amount of kappa in the course of 5 more fissions. As the mean number of particles at the 21st fission was calculated to be 0.3 per cell, there should be a total of 6.9 × 0.3, or 2.1, particles of kappa among the 32 resulting animals, or 0.065 particles per animal. The value of $e^{-m}$ when m = 0.065 is 0.94, which agrees well with the observation that 93% of the animals were irreversibly sensitive after 26 fissions. It may therefore be concluded that the observations agree completely with the assumption that kappa is being distributed essentially at random during fission.

The other main assumption, that one particle of kappa in an animal is all that is required to enable that animal to transform eventually into a killer when its reproduction is suppressed, has also been tested experimentally by Preer (7). The principle involved in the test is based on the observation, set forth above, that the percentage of animals incapable of reverting to killers increases with the number of fissions during rapid reproduction. As has been shown, this is due to the progressive decrease in concentration of kappa. It has been assumed that the animals incapable of reverting to killer have no kappa. Suppose, however, that animals cannot revert to killers if they have fewer than some higher number of particles of kappa, such as 10 for example. Then the data would mean that the percentage of animals with less than 10 particles of kappa increases with the number of fissions during rapid reproduction. These two alternatives lead to very different expectations under certain circumstances. If one starts a culture with an animal known to have a concentration of kappa slightly greater than the required minimum for reversion, and allows this animal to reproduce rapidly, the maximum number of particles any one of the progeny will contain will decrease as the number of fissions increases, until practically no animals contain more than one particle; but there will always be a certain proportion containing one particle, this proportion progressively diminishing (though the total *number* of animals with one particle actually increases). In view of this situation, if 10 particles were required for reversion, the proportion of cultures containing any animal capable of reversion would decrease as the period of rapid fission increased; but, if one particle is enough for reversion, the proportion of cultures containing any animal capable

of reversion will remain the same no matter how extended the period of rapid fission. For the test to be valid, however, *all* the progeny in each culture must be retained for observation. Preer performed such an experiment and found that the proportion of cultures containing at least one animal capable of reverting to killer remained the same when the period of rapid fission increased. Consequently, it must be concluded that a single particle of kappa in an animal is enough for it to revert to killer when fission is later suppressed.

few particles of kappa. Preer's method might then be used to compute the mean number of particles of kappa after various periods of reproduction at known rates; and in this way the rate of increase of kappa and the final concentration of kappa in killers might be computed. The prerequisite for this type of study is to have methods of starting animals off with very small amounts of kappa. Two such methods have been worked out. This first will be described in the present section and the other in Section IV.
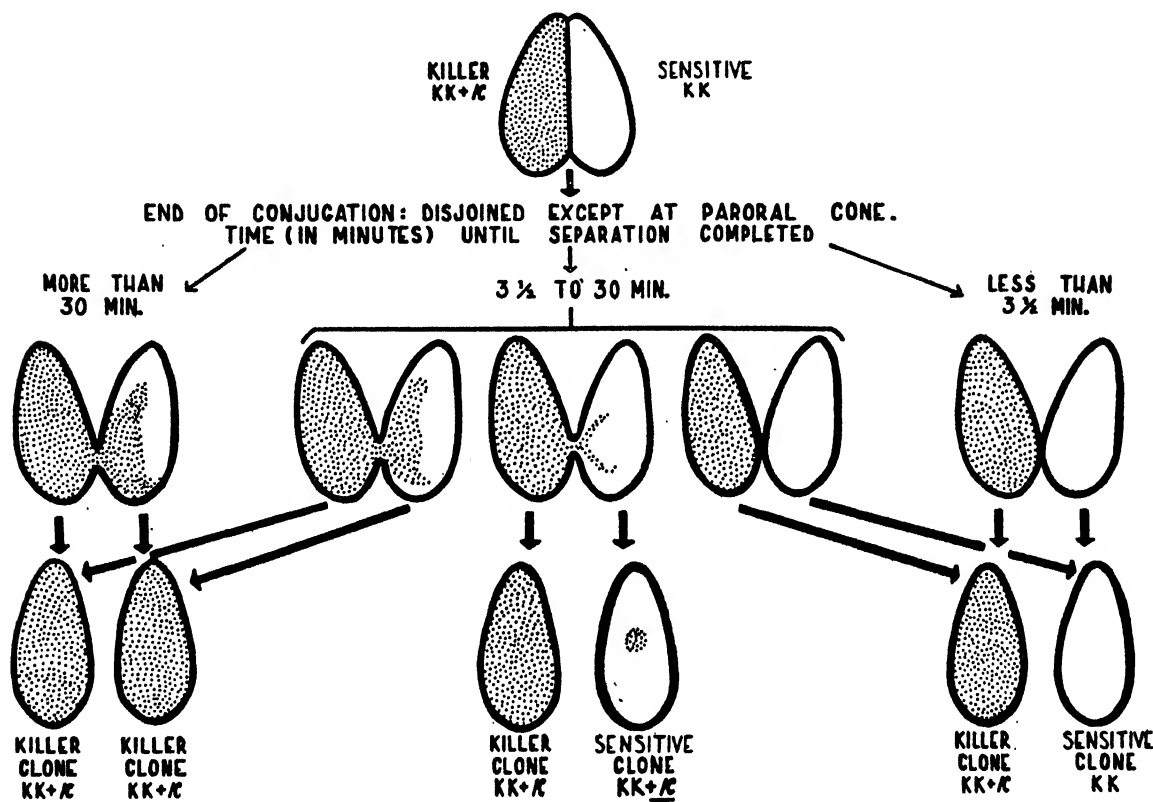


Fig. 3. Effects of transfers of different amounts of cytoplasm between mates in crosses of *KK*-plus-kappa killers by *KK* sensitives. The amount of cytoplasm exchanged is roughly proportional to the time the paroral bridge persists after the conjugants have separated elsewhere.

## III. CONTROL OF THE CONCENTRATION OF KAPPA BY VARIATIONS IN THE AMOUNT OF CYTOPLASM TRANSFERRED DURING CONJUGATION

The methods set forth in the preceding section are applicable only to material in which kappa cannot increase as rapidly as the animals can. This is *not* the condition in stock 51 of variety 4. This killer stock, which was the material used in practically all of the genetic work, can reproduce twice as fast as the killers of variety 2; yet it remains pure killer, nevertheless. For material of this kind, therefore, different methods must be devised. If possible, the procedure should be reversed: cultures should be started with animals containing only one or a

The first method has already been described in an earlier paper (*12*). It consists of taking advantage of the natural variation that occurs in the amount of cytoplasm transferred between mates during conjugation and in utilizing a readily observed index of this variation. The general method is indicated in Fig. 3. The amount of cytoplasm exchanged at conjugation is roughly proportional to the time during which a visible cytoplasmic bridge (a fusion of the "paroral cones") connects the conjugants after they have separated elsewhere. Most conjugant pairs separate at the bridge within 3.5 minutes after the process of separation has been completed elsewhere. In certain crosses, however,

this bridge remains for longer periods, in the extreme cases remaining permanently and resulting in the eventual formation of a double animal. More commonly, however, the bridge breaks after a longer or shorter period of time. In the cross of the killer stock 51 to the *KK* sensitive stock 47, the sensitive member of a pair of conjugants never gave any evidence of having received kappa if the mates separated at the paroral bridge within 3.5 minutes after separation elsewhere. If this bridge persisted for more than 30 minutes, the sensitive member of a pair invariably produced promptly a clone of killers, showing that it had received an ample supply of kappa. When the bridge persisted for intermediate times, any one of three results occurred. Two of these were the results already described—that is, either no evident transfer of kappa or transfer of an abundance of kappa. The third result, however, was different from anything that had previously been known. The original sensitive conjugant produced a sensitive clone, yet the members of such clones contained kappa! The detailed proof of the presence of kappa in these clones has been published previously (*12*); here it need only be recalled that the presence of kappa in sensitive animals could be detected by their production of killer offspring when they underwent an internal self-fertilization process known as autogamy. For the moment, the theoretically important question of why this transformation occurs at autogamy will be put aside; it will be considered later. The fact that the presence or absence of kappa in sensitives can be tested in this way provides a useful tool for analysis, as will appear at once.

The sensitive clones, derived from conjugants into which small amounts of kappa had been introduced, consisted of animals of two kinds: some contained kappa, others did not; the former could produce killer progeny at autogamy while the latter could not. Animals in which kappa no longer existed arose sometimes at the very first fission of the conjugants, sometimes at the second fission, sometimes later. This situation is comparable to the one described in the preceding section; hence, the frequency with which animals lacking kappa arise can be used as a measure of the mean number of particles of kappa per animal. In order to obtain at fission one daughter animal with kappa and one entirely lacking kappa, the parent animal would probably have 7 particles of kappa or less. With 7 particles there would be less than 1 chance in 100 that all 7 would go into one daughter animal and none into the other. With fewer particles of kappa the probabilities would be correspondingly greater. The technique employed is thus adequate for obtaining animals with initially very few particles of kappa.

There is, however, a serious practical difficulty in trying to apply this method. The only way to select a sensitive conjugant into which the desired small number of kappa particles had been introduced would be to select one in which the paroral bridge had lasted between 3.5 and 30 minutes. Among the conjugants selected in this way, however, there would be many that contained no kappa at all and many that contained too much (i.e., more than 7 particles). One would have to try a considerable number of conjugants in order to be reasonably sure of finding one of the kind desired. As each conjugant selected for study would give rise to a large experiment in itself, it would be impracticable to sample very many animals in this way.

Actually, the experiments were performed before the possibilities for analysis were realized. With other purposes in mind, a considerable number of conjugants was examined, but only a few daily isolation subcultures of each were followed. In spite of these limitations, some information significant for present purposes was obtained. Among the many conjugants examined, four were found to have an amount of kappa appropriate for studies of this kind. The four animals resulting from the first two fissions of each of these conjugants were separately cultivated as daily isolation lines of descent; that is, from among the progeny produced each day by a single animal, one was reisolated to continue the line of descent. As the animals reproduced rapidly, each one yielding from 32 to 128 progeny in the course of a day (until the line became old and weak), the method of cultivation consisted of a series of chance daily selections of one animal from among its 31 to 127 sister animals in each line of descent. These details are important for understanding the results.

Two types of test were applied to the daily isolation lines of descent. In the first type of test, which was made nearly every day, the animals left over at the time of a daily isolation were allowed to go through a few more fissions and were then tested to find out whether they were at that time killers or sensitives. In the second type of test, which was performed as frequently as time permitted, the animals left over after a daily isolation were again allowed to go through a few more fissions and were then starved so as to induce them to go into autogamy. A number of autogamous animals were then separately isolated, and each was grown into a culture, which was tested to ascertain whether it was killer or sensitive. In this way, it was possible to discover what proportion of the animals was capable of becoming killers after autogamy and what proportion was unable to do this because of a complete absence of kappa. Autogamous cultures which failed to become killers were kept under observation until after a second autogamy, for in some cases two successive autogamies were required to transform sensitives into killers. Cultures were classified as lacking kappa if they failed to produce killers after two successive autogamies.

Of the 15 lines of descent (one of the original

16 died early) derived from the four conjugants, 5 remained permanently sensitive and 10 eventually turned into killers. (In the original paper, the question of whether the 10 lines that became killers did so in the absence of autogamy was left open. Recent results prove that this does happen.) One might suspect that a very fundamental difference distinguishes lines that differ so much in fate. Actually, however, the data indicate that this extreme difference in end results follows simply from the chance distribution of kappa at fission, and that the two kinds of lines were initially identical. In order to understand this conclusion, the data on representative lines must be examined in some detail.

Line 3b1a is an example of a line of descent that remained permanently sensitive. Tests on groups of left-overs that had been sent into autogamy gave different results at different times. The first group of autogamous progeny was obtained when the parent line had been through only 18 fissions: 8.5% of this group were unable to transform into killers; hence, at this time, 8.5% of the animals in the parent line lacked kappa. Employing the Poisson series, as in Section II above, it may be calculated that there was at this time a mean of 2.5 particles of kappa per animal. A second autogamous group was obtained 6 fissions later, when the line was 24 fissions old. In this group, all of the autogamous cultures were unable to become killers; kappa had completely disappeared. The same result was obtained in a third group of autogamous animals when the line was 51 fissions old. In this line, therefore, kappa disappeared between the 18th and 24th fissions—that is, after the fourth daily selection of a single animal to continue the isolation line. In view of the fact that the mean number of particles of kappa per animal prior to this time was only 2.5 and an appreciable proportion (8.5%) of the animals lacked kappa entirely, the selection of an animal with no kappa to continue the daily isolation line could readily occur by chance. The other four lines of descent, which remained permanently sensitive, can be similarly explained. In each, the data on autogamous groups show that a very small number of particles of kappa was present at the start, and that the mean number per animal remained low during this period of rapid reproduction. A kappaless animal was selected to continue the line at the 4th to 18th daily selections in different lines of descent. This range of variation is consistent with expectations based on chance alone.

Line 2b2a is an example of the other sort of line, in which kappa was permanently maintained and in which eventually the daily isolation line itself transformed into killers. After fissions 25, 48, and 66, respectively, autogamous groups were obtained from the left-overs from the isolation lines. In each of these three groups, from 10 to 18% of the autogamous animals were shown to lack kappa. By equating these values to $e^{-m}$, the mean

number of particles of kappa per animal may be computed as approximately 1.7 to 2.3 during the period in which these three tests were made. Thus the number of particles of kappa per cell did not vary significantly during a period of seven days in which 41 fissions took place at the rate of 5.9 per day; in other words, kappa was increasing at a rate of approximately 6 doublings per day. As in the lines which eventually lost kappa entirely, variation in the number of particles per animal seems to be due entirely to chance distribution. From observations of this sort (though analyzed less precisely), it was concluded in 1945 (*12*) that the maximal rate of multiplication of kappa is approximately the same as the maximal rate of multiplication of the paramecia themselves.

Under certain conditions, however, kappa multiplies more rapidly than the animals, and this results in sudden and marked increases in the concentration of kappa. This is illustrated by further observations on line 2b2a. Although this line was sensitive in every test until the 76th fission, the next test (made after the 90th fission) showed that the line had become resistant (though not a killer), and at the following test (after the 95th fission) it was a killer. Thereafter it remained a killer. These observations indicate that the concentration of kappa began to increase before the 90th fission and reached a level sufficient to determine the killer character by the 95th fission. Further evidence of a higher level of kappa concentration was obtained from tests on an autogamous group, obtained when the line was 86 fissions old. Every autogamous culture transformed into killers; hence, at this time all members of the parent line contained kappa, and the mean number of particles of kappa per cell had risen above 7.

To what is this increased concentration of kappa in animals of line 2b2a to be attributed? Is it due to the accumulation of kappa as a result of chance variations in distribution at fission, or is some other process involved? Examination of the daily fission rate of the line seems to give the clue to the solution of this problem. At the time of the 86th fission, its reproductive rate dropped from over 5 fissions per day to 4.4 fissions per day. The first day on which only 4 fissions occurred was the day on which the line became resistant. The next day it became a killer. Decrease of fission rate and transformation into killers seem to be correlated in general. In 8 of the 10 lines which transformed from sensitives into killers during vegetative reproduction, this transformation occurred either on the first or the second day on which the fission rate dropped to 4. In one of the other two lines it occurred slightly later, and in the other line slightly earlier.

In view of what has already been set forth, the significance of these observations should be apparent. As brought out previously, when the animals multiply at their maximum rate under the condi-

tions of cultivation—that is, at a rate of 6 fissions per day—the amount of kappa per animal remains practically constant; hence, the rate of kappa increase and the rate of animal increase are for all practical purposes identical under these conditions of optimal growth. If the rate of increase of kappa is maintained at this high level, or nearly so, when the fission rate of the animals decreases during old age, then the amount of kappa per animal must increase. On a day when the animals divide only 4 times, kappa may thus be able to double itself 6 times, thereby possibly increasing the amount of kappa per animal by a factor of 4.

The conclusion that the concentration of kappa rises when the fission rate of the animals decreases is supported by several other lines of evidence. In the first place, as shown in Section II, Preer has demonstrated in variety 2 that the rate of increase of kappa is less than that of the animals when the animals multiply rapidly, but is more than that of the animals when the animals multiply slowly. Secondly, killer cultures which are incapable of rapid reproduction are extremely powerful killers, much more so than normal cultures of killer stock 51. (Such slowly multiplying killer clones of low viability arise commonly in the $F_2$ generation following crosses of killers to other stocks.) A single animal of such a clone will kill hundreds of sensitives in a period in which a normal killer animal will kill only a dozen or less. A third observation, which has not yet been analyzed experimentally but which is a matter of routine laboratory experience, is that overfed and rapidly multiplying killers show much less killing action than killers that have begun to exhaust their food supply and cease to reproduce. Finally, the effect of autogamy in transforming sensitives with kappa into killers may be due to the fact that on the day in which autogamy occurs the fission rate drops from 5 or 6 to 1 or 2. To this we will return later. Altogether, these lines of evidence indicate strongly that the concentration of kappa rises in animals that reproduce at a submaximal rate.

Two aspects of this matter remain to be investigated. First, what is the effect of temperature? Fission rate varies with temperature, as is well known. Does the rate of increase of kappa vary in the same way? Or are the two rates unequally affected by temperature? In the work discussed above, increase of kappa concentration followed from decrease of fission rate only when the rate was decreased by inadequate food, aging, constitutional factors, or the occurrence of interfering internal processes (autogamy). Whether similar results would follow from fission-rate decreases brought about by reduction of temperature still remains unknown. Second, the concentration of kappa certainly cannot go on increasing indefinitely when the animals are multiplying slowly. In the extreme case, when the animals are not multiplying at all, this

would lead to the absurd conclusion that soon the killers would be converted completely into kappa. There must be some limiting factor, perhaps the amount of available precursor, which sets a limit beyond which the concentration of kappa cannot rise.

The preceding discussion of the relation of fission rate to kappa concentration provides a basis for understanding how it happens that, among a group of animals containing small numbers of particles of kappa, some produce daily isolation lines of descent that lose kappa and remain permanently sensitive while others give rise to lines that eventually transform into killers. At first, when they are young (i.e., in the period following their origin at conjugation), the animals reproduce at their maximal rate and kappa reproduces at approximately the same rate, keeping the concentration of kappa at about the same average level. During this period inequalities in kappa concentration arise, however, as a result of the random distribution of kappa at fission, and a small proportion of animals thereby receive at their origin no kappa at all. As only one animal is selected each day to perpetuate the line, there is always a chance of selecting an animal lacking kappa. When this happens, as it does occasionally, the line is thenceforth destined to remain permanently sensitive and it cannot produce killer progeny at autogamy. In the 15 lines of descent in the experiment under consideration, there were about 200 opportunities for this to happen, and it happened 5 times. This is about the frequency expected by chance if there had been a mean of about 4 particles of kappa per animal. This estimate of kappa concentration agrees sufficiently well with that based on other methods of estimation.

Later, when the lines have grown older, the situation changes. With age comes a declining fission rate (2, 8, 15) and, as has been pointed out, kappa concentration rises under these conditions. Lines of descent that have by chance retained kappa during the period of rapid fission must therefore experience during middle and old age an increase of kappa concentration. This has two consequences. As the concentration of kappa rises, (1) the chance for the production at fission of an animal lacking kappa decreases until it becomes, for all practical purposes, zero; (2) the character of the line changes from sensitive to killer. If a line of descent can hold on to kappa until it begins to grow old and feeble, it will then transform into a killer. The difference between lines of descent that remain permanently sensitive and lines that eventually transform into killers is accidental only and depends merely on the random distribution of kappa at fission during the early vigorous period of life when the level of kappa concentration remains low.

In view of what has already been set forth, it is possible to estimate the number of particles of kappa present when a line has transformed from

sensitive into killer. In the case of line 2b2a discussed above, calculations indicated that there were only about 2 particles of kappa per animal at the time of the 66th fission. Up to this time the line had been reproducing at a rate of about 6 fissions per day and the concentration of kappa had remained constant. Hence, kappa was doubling itself about 6 times per day. The rate of fission then dropped for 4 days to 5 fissions per day. If kappa continued to double itself 6 times per day, this would have brought the concentration up to 32 particles per animal. On the following day, when the fission rate dropped to 4, there would have been another fourfold increase in concentration, bringing the number of particles up to 128 per cell. The following day 5 fissions occurred, doubling kappa concentration again and bringing the concentration up to 256 particles per animal. At this stage, the culture became a killer. This estimate of 256 particles of kappa in a killer agrees with the results of Preer on another variety, although the calculations were made in a different way. This estimate for variety 4 is of course only a rough first approximation. However, similar calculations made on other lines of descent lead to essentially the same result. For example, line 2b2b became a killer on the day when its cumulative fission rate reached a total of 8 fissions less than would have been accumulated at a steady rate of 6 fissions per day. This would have permitted a 256-fold increase of kappa concentration if kappa had multiplied steadily at a rate of 6 doublings per day. Detailed data for other lines lead to similar estimates.

There remains for consideration the theoretically important question of the role of autogamy in transforming sensitives with kappa into killers. Reproduction by fission cannot occur while animals are undergoing the prezygotic and fertilization stages of autogamy and conjugation, and these stages occupy about ¼ day at 27° C. Moreover, the initiation of these processes is preceded by a period of some hours during which feeding is reduced and growth retarded; and the first postzygotic fission occurs about ½ day after fertilization, the second postzygotic fission following the first by an interval somewhat longer than the usual interfission period of four hours. Consequently, on the day that autogamy or conjugation occurs, animals go through not more than 2 fissions, while purely vegetative animals go through 6 fissions. If this sort of retardation in the fission rate had the same effect on kappa concentration as retardations due to old age or inadequate food, the concentration of kappa could increase by a factor of $(2)^4$, or 16-fold, when animals with little kappa undergo fertilization.

In a previous paper (*12*) it was shown that no ordinary genetic features of autogamy could be the cause of the change of phenotype. It was suggested, however, that the disintegration of the macronucleus might release kappa which had been

stored in it and could not escape into the cytoplasm in any other way. Preer's work, cited in Section II, seems to eliminate this possibility, for he was able to rid variety-2 animals of kappa during purely vegetative reproduction by means of rapid growth alone, and kappa did not reappear at subsequent fertilizations when the macronucleus disintegrated. No other way of explaining the effect of autogamy is now evident except that it is a means of increasing the concentration of kappa simply as a result of the accompanying retardation of fission rate.

This explanation is consistent with another observation; namely, that sometimes one autogamy is not enough to accomplish the transformation of killers into sensitives, but that a second autogamy will succeed where the first one failed. The calculations made above indicated (1) that the order of magnitude of kappa particles required for an animal to be a killer is about 250 and (2) that the retardation of fission accompanying autogamy might be expected to bring about a 16-fold increase in kappa concentration. Hence, if there were only 1 particle of kappa in an animal, one autogamy would increase the number to 16, but two autogamies would bring it up to 256. The former would certainly be inadequate, the latter adequate. In agreement with this, there has never yet been observed a culture which became killer after a later autogamy if it failed to become killer by the second autogamy.

The three known conditions under which sensitives containing kappa can be transformed into killers thus seem to operate through a common mechanism. Inadequate food, old age, and autogamy all involve reduction of fission rate, and it is apparently this that enables kappa to increase more rapidly than the animals and thereby to bring about a net increase in the concentration of kappa in the animals.

## IV. CONTROL OF THE CONCENTRATION OF KAPPA BY MEANS OF TEMPERATURE

Until very recently, there was no known exception to the rule that the killer race 51 reproduces absolutely true to the killer character. A short time ago, however, cultures of this stock stored at 10° C. for a period of 6 months were found to contain some sensitive animals, and, during even shorter storage periods, some cultures of hybrid killers turned completely into sensitives. This accidental discovery led to an extensive investigation of the effects of temperature on killers. Although this investigation is still in its early stages, it soon appeared that dramatic results follow exposure to high temperatures. The possibilities opened up for further study are so exciting that a preliminary report seems worth while at this time.

When animals of the killer stock 51 are placed at 38° to 39° C., some fail to divide at all, some divide only once, and a few may divide twice; but all die in 3 to 3½ days. If they are removed from

38.5° C. after shorter exposures and returned to the standard cultivation temperatures of 24° to 27°, the effects observed are proportional to the period of exposure to 38.5°, as set forth in the following paragraphs.

After an exposure of 36 hours or more to 38.5° C., all killers are transformed into sensitives and those that survive yield exclusively sensitive clones. If these clones are allowed to undergo one or more autogamies they still remain pure sensitives. One exposure to high temperature brings about a complete and irreversible change in character from killer to sensitive. Thus, by every available standard, exposures of 36 hours or more result in the complete disappearance of kappa.

After exposures of 24 hours to 38.5°, most of the animals (77 to 87%) were exactly like those that had been exposed for 36 or more hours. They were sensitive and unable to produce killer progeny; they completely lost kappa. The remaining animals retained some kappa, for they were able to produce some killer progeny after autogamy. From the Poisson series, it may be calculated that about 90% of the animals that retained kappa had at the end of the heat treatment only a single particle of kappa, and over 90% of the remainder had but two particles. An exposure of 24 hours to 38.5° C. is thus a practical means of obtaining animals containing only 1 or 2 particles of kappa.

When killer animals are exposed to 38.5° for only 12 hours, they are likewise converted into sensitives; but all are capable of producing killer progeny after autogamy. This shows, therefore, that the high temperature has not destroyed all the kappa, but has destroyed enough to alter the character of the animals from killer to sensitive. From each of the treated animals 4 to 8 subcultures were started with the products of the first 2 or 3 fissions and each of these subcultures was maintained by the daily isolation culture technique. Periodically, the animals left over after one was selected to continue the line were tested to discover whether or not the line had transformed into killers (in the absence of autogamy). The rate of reproduction of the isolation lines was not uniformly high. As a result, some lines of descent transformed into killers by the time they had gone through 24 fissions. Others transformed into killers at later times, as indicated by tests made after the 50th, 65th, 80th, 92nd, and 105th fissions, respectively. Still other cultures, however, failed to transform into killers at any of these tests, and when they were again allowed to produce autogamous cultures no killers arose in them. Hence, as in the comparable situation described in Section III, in these lines animals lacking kappa had by chance been selected to continue the daily isolation lines. Thus, while a 12-hour exposure results directly in no animals lacking kappa, the mean number of particles per animal is so much reduced that kappaless animals can arise as a result

of the random distribution of the particles to the daughter animals arising at subsequent fissions.

The observations on the progeny of animals exposed to 38.5° for 12 hours have established two facts that were suspected but inadequately demonstrated in the work described in Section III. First, it was proved that sensitives containing kappa could transform into killers in the absence of autogamy. Second, it was shown that there exists an intermediate condition between sensitive and killer, a condition in which the animals are neither sensitives nor killers: they are resistant nonkillers. This phenotype develops shortly before the animals transform into killers; this indicates that it is due to concentrations of kappa intermediate between the higher concentration required for the killer character and the lower concentration that results in sensitivity.

A series of characters thus seems to be determined simply by the concentration of kappa. When no kappa is present, the animals are pure sensitives; they are unable to produce killers in the course of either fissions or autogamy. When there are present very few particles of kappa, the animals are likewise sensitive but they can by chance produce some vegetative progeny that lack kappa completely and they can also produce killers when the fission rate is reduced. With a slightly higher concentration of kappa, the animals are still sensitive and can still produce killers under the same conditions but they can no longer produce kappaless progeny with a detectable frequency. A further rise in the concentration of kappa results in the loss of sensitivity: the animals become resistant nonkillers. When the concentration of kappa is still higher, the animals are normal killers and reproduce true to this character during vegetative and sexual reproduction.

Exposures of 6 hours or less to 38.5° C. bring about no change of character in the organisms: all retain the killer character. Although some kappa may possibly be destroyed at this exposure, if so, the amount destroyed is too small to bring about a detectable change in the characteristics.

The foregoing results show that there is a quantitative relation between length of exposure to 38.5° and the amount of kappa destroyed. The detailed relations are now under investigation, with a view to constructing a heat-inactivation curve. Such a study should provide means of attacking a number of important problems. It should provide another method of calculating the concentration of kappa, not only in killers but also in killers of different strength, in resistant nonkillers, and in sensitives. This may be attacked from both ends: by measuring the exposures required to reduce strong killers to each condition dependent upon a lower concentration of kappa; and by measuring the exposures required to destroy all kappa in animals with each of these grades of character. In like manner, if one uses

the heat technique to start with animals containing only one or any other known number of particles of kappa, it should be possible to measure the effect of autogamy and other conditions that increase kappa concentration by measuring the exposures required to cancel their effects. Perhaps it may be possible to discover from the inactivation curve, particularly if other physical agents bring about the same effects, whether the particles of kappa are single molecules or molecular aggregates. Finally, the same methods may be successful in attacking similar problems concerning other cytoplasmic factors in Paramecium and in other organisms. In our present state of hopeful ignorance, the prospects for the future of this technique look rosy indeed.

## V. Discussion

### The Rate of Increase of Cytoplasmic Factors

The evidence already in hand permits the conclusion that cytoplasmic factors do not all reproduce at the same rate. This is brought out strikingly by a comparison of kappa in variety 4 with the corresponding cytoplasmic factors in variety 2. In stock 51 of variety 4, kappa can increase at a rate equivalent to 6 doublings per day, while the corresponding factor in stock G of variety 2 seems to be unable to increase at a rate higher than the equivalent of 2 doublings per day. Preer (7) has evidence that the cytoplasmic factors for killing in different kinds of killers of variety 2 reproduce at different rates. The rate in stock G seems to be the highest, while stocks H, 36, and 50, which are different kinds of killers, contain cytoplasmic factors for killing which reproduce even more slowly. Of more interest than the absolute maximum rate of increase of kappa in different stocks is the relation between the rate of reproduction of the animal and the rate of increase of kappa. In variety 4, the animals can undergo fission 6 times per day and kappa can increase at the same rate; but, when the concentration of kappa is low, kappa can increase more rapidly than the animals, if the latter are by certain conditions prevented from multiplying at their maximum rate. In variety 2, on the other hand, the corresponding kappas cannot increase as fast as the animals multiply, when the latter reproduce at their relatively low maximum of 3.5 fissions per day. Yet, even in variety 2, kappa increases faster than the animals when the latter are made to multiply slowly. There is thus no necessary agreement between the rate of reproduction of animals and genes and the rate of reproduction of cytoplasmic factors.

What is the cause of the different rates of increase of different cytoplasmic factors? The different rates may be characteristic of the cytoplasmic factors themselves, and might be manifested in any genetic background in which they could grow at all. On the other hand, the same cytoplasmic factor might increase at different rates in different genetic backgrounds. This problem is under investigation, but the solution is not yet in hand.

### The Concentration of Cytoplasmic Factors

The results set forth in this paper make it reasonably certain that, with respect to the cytoplasmic factor for the killer character, the normal concentration is always extremely low, of the order of magnitude of 100 to 300 particles per animal. This fact, combined with the demonstrated approximately random distribution of the particles during the process of fission, suggests (but does not prove) that the particles are scattered throughout the cytoplasm. If so, there would be about one particle of kappa per 1000 $\mu^3$ of cytoplasm. Even if this figure turned out to be in error by as much as a factor of 10, or even more, the particles of kappa would probably each be effectively isolated from all the others. Whether each particle of kappa is a single molecule, or a group of molecules that behaves as a unit in the process of distribution at fission, cannot yet be answered from any experimental evidence. In either case, it would seem that the biochemistry of such a cytoplasmic factor may be of a sort quite different from that usually visualized, in which the laws of mass action are assumed to operate. It may well be that, for the study of the biochemistry of cytoplasmic factors, there will need to be developed a biochemistry that can handle the reactions of single isolated molecules or aggregates of molecules. This offers no small challenge to the ingenuity and imagination of biochemists with an appreciation of the importance of systems of this sort. When one considers at the same time that our present information indicates a cytoplasmic factor intervening between each kind of gene and the cellular processes it controls, one may imagine that the cytoplasm contains perhaps thousands of different kinds of cytoplasmic factors, each one present in extreme dilution and operating biochemically as a single isolated molecule or molecular aggregate.

The discovery of the low cellular concentration of kappa makes intelligible another recent discovery. When one studies simultaneously the inheritance of two or more nonallelic characters, each of which depends upon a cytoplasmic factor, it is found (13) that the different kinds of cytoplasmic factors are separately transmissible from one mate to the other across the paroral bridge. In any particular pair of conjugants one kind of factor may be transferred while another kind is not; or both kinds may be transferred, or neither. This could happen only if one or the other of two conditions existed, as Tatum pointed out to me. Either the amount of cytoplasm transferred must sometimes be extremely minute, or the cytoplasmic factors must be present in extremely small numbers in the animals. In view of the results presented in this paper, the latter seems to be the correct explanation. The low concentration of cytoplasmic factors thus makes it

possible for them to be recombined, in a different way from the genes but with analogous results.

### Dauermodifikationen

There is a remarkable resemblance between some of the results set forth in the present paper and some of the perplexing phenomena discovered by Jollos (3) and interpreted as *Dauermodifikationen*. In a number of cases Jollos found that hereditary variations, thought to have been produced by environmental means, disappeared or reverted to the original condition as a result of autogamy or conjugation. He also reported that sometimes two or more autogamies would have to occur before reversion took place. Without going into an account of Jollos's attempts to explain this observation, which has been one of the most puzzling features of his work, we may point out that the present work with kappa seems to provide a simple and reasonable explanation. The transformation of sensitives into killers at autogamy is due not to any genetic feature of autogamy, but simply to the temporary cessation and retardation of the process of fission, which allow the concentration of kappa to rise. When kappa is present in very low concentrations, it takes two autogamies to raise the concentration to the level that can bring about a change of character. It therefore seems possible that Jollos's hereditary changes resulted from a lowering of the concentration of some cytoplasmic factor and that the subsequent increase in concentration brought about by one or two autogamies served to restore the concentration to the original level. In this connection, it is interesting that some of Jollos's *Dauermodifikationen* were produced by exposure to high temperature, which is now known to inactivate and destroy kappa. Further, Jollos claimed and showed that restoration of the original character could also occur during purely vegetative reproduction; and the level of kappa concentration can also rise during periods of vegetative reproduction, when the fission rate is reduced. Another feature of Jollos's observations is his finding that some *Dauermodifikationen* were both produced and lost in a series of quantitative steps or grades. This seems to have its parallel in the step-like progression from sensitive to resistant to killer, a progression based on progressive changes in concentration of kappa. Finally, Jollos was able to produce *Dauermodifikationen* only in certain races of Paramecium; and it is now known that cytoplasmic factors are evident only in the races of certain varieties. Many of Jollos's observations are thus in agreement with the results of recent researches, though the interpretations now given to such observations are radically different.

### "Aging" and the Life Cycle in Protozoa

It has long been known that at least some Protozoa manifest a life cycle starting with conjuga-tion. There follows a period of immaturity in which the organisms cannot mate, a period of sexual maturity, and a period of senescence ending in death. During the first two periods the organisms multiply rapidly, but the rate of fission may decline progressively during senescence. In *P. aurelia,* the normal periodic occurrence of autogamy keeps vitality high; but if autogamy is avoided, the fission rate eventually falls and the animals sooner or later die (2, 8, 15). If autogamy occurs early in the period of senescence it usually restores vitality; later there may be only a partial restoration or only in a fraction of the animals (2, 8). In Spathidium, Woodruff and Spencer (16) have shown similar effects of conjugation and, further, that sometimes two or more successive conjugations are required to restore vitality fully.

This is a large and complex subject, and an extended discussion would be required to deal with it adequately. Here it may merely be indicated that there is at least a possibility of analyzing it along lines developed in this paper. The period of immaturity may be a period in which the concentration of a cytoplasmic factor involved in the determination of mating type is being slowly built up after its depletion during the process of conjugation. The period of senescence may be due at least in part to the gradual reduction in concentration of a cytoplasmic factor that cannot increase as fast as the cells but that is essential for life. The fact that mortality at autogamy in *P. aurelia* increases with the time since the last previous fertilization (1, 6) might be used, like Preer's data in Section II, to compute the rate of increase and number of particles of such a hypothetical factor. This mode of approach to the classical problem of the life cycle in Protozoa would seem to be worth pursuing, though I am well aware of some difficulties in applying it.

### Genetics of Other Microorganisms

Studies on heredity in bacteria or viruses are ordinarily studies on inheritance within a clone. As has been brought out in other papers in this Symposium, workers on such forms have an understandable desire to relate their results to the genetics of higher organisms. Persistent hereditary variations are spoken of as mutations, and, in spite of all caution, we are only too apt to think of them as gene mutations or whatever their analogue may be in organisms of this kind. This way of thinking may be correct, at least in many cases. However, work with the killer paramecia serves to show, among other things, that hereditary changes are not always of this kind. The change from killer to sensitive, or the reverse, can take place not only in the absence of gene mutation, but even in the absence of mutation of the cytoplasm factor; such transformations result simply from changes in the concentration of the cytoplasmic factor. Moreover,

these changes in concentration, with their accompanying phenotypic changes, may persist for many cell generations, certainly up to 100 cell generations. Possibilities of this sort might well be taken into consideration in the interpretation and planning of work on the genetics of organisms of this sort. Such a point of view is particularly indicated when dealing with a series of variations which form an apparently quantitative series, comparable to the series strong killer, normal killer, resistant, and sensitive. Some of the characters reported upon in other papers of this Symposium do in fact appear to form quantitative series of this kind.

### Implications for Certain Problems of Developmental Differentiation in Higher Organisms

The problem of developmental differentiation is, of course, always in our minds; until it is solved, attempts to relate it to current studies may have some value. A developing multicellular organism is, as has often been pointed out, comparable to a clone of microorganisms. The cell-progeny of the fertilized egg constitutes a clone comparable to the progeny of a single microorganism. Tissue cultures have shown that some of the cellular differences arising among the progeny of the fertilized egg are persistent and irreversible, some are not. The appearance of comparable irreversible and reversible differences among the progeny of a single microorganism raises the question of whether any of the mechanisms operating in the microorganisms might be similar to some of those operating during development from the egg. Differences with respect to killing, resistance, and sensitivity may arise within a clone, some of these being irreversible. In this case, the mechanism is clearly one of changing the cell concentration of a cytoplasmic determinant. The changes are brought about in two ways, which might also operate in the case of development from the egg. The first is through a difference between the cytoplasmic factor and the cells in their rate of increase. The second is through an inactivation or destruction of cytoplasmic factor by certain conditions external to the cells involved. In either case, the cellular concentration of the cytoplasmic factor may be brought to so low a level that cells arise which by chance lack the cytoplasmic factor entirely. If the cytoplasm of the egg contains a number of diverse determinative cytoplasmic factors which, like kappa, are not producible by the genes, then any cell that lacked such a factor would differ irreversibly from cells that contain it. Further, the characters of those cells that contain the factor might differ in dependence on the cellular concentration of the factor. Any cytoplasmic factors that were essential for the life of each cell would have to be present in sufficient numbers and would have to multiply at a high enough rate so that they could not be lost from any cell. On the other hand, factors that underlie cellular differentiation could be

present in low numbers or could increase at rates less than the maximal rate of cell proliferation. In the former case, there would arise by chance some cells which lacked the factor; in the latter case, there would arise gradients and fields correlated with the rate of cell division in a particular region.

These possible relations of cytoplasmic factors to development appear also in the important studies of L'Héritier and his co-workers (4, 5) on a cytoplasmic factor or "genoid" in Drosophila. The results on the genoid of Drosophila are similar in many ways to the results on cytoplasmic factors of Paramecium.

### Hypothesis of the Bipartite Structure of the Gene

In previous papers (11, 12), the conception of the gene as consisting of two parts was proposed as a working hypothesis. One part, localized in the chromosomes, was assumed to function primarily in the process of self-duplication; the other part, occurring both in the cytoplasm and bound to the first part in the macronucleus, was assumed to be the physiologically active part of the gene, entering into the determinative processes of cellular physiology. Each cytoplasmic factor of Paramecium was assumed to be the free and active part of a gene. Although still capable of union with their corresponding gene-parts in the macronucleus, the cytoplasmic factors were held to be absent from the micronuclei and incapable of union with the micronuclear genes.

Two lines of experimental evidence were given in support of the union of kappa with macronuclear genes. Neither of them now appears tenable in view of the newer knowledge reported in this paper. The fact that the rate of increase of kappa may be either greater than, less than, or the same as the rate of increase of the genes practically rules out the possibility that kappa is multiplied as a part of gene $K$, which was assumed to be the case for sensitives that contain very little kappa. Both lines of evidence therefore require reinterpretation. One of them—the evidence from the transformation of sensitives with kappa into killers at autogamy—has already been satisfactorily reinterpreted above (page 243). The other line of evidence—from the production of pure sensitives by killers following macronuclear regeneration—is not so easily reinterpreted at present. There are, however, certain alternative interpretations that can and will be tested by further investigations.

While no experimental evidence remains in support of the present combinability of kappa and gene $K$, there still exists a strong indication that the cytoplasmic factors found in certain varieties of Paramecium correspond to parts of the genes in other varieties of Paramecium. As pointed out in an earlier paper (11), in one group of varieties, each character seems to be based upon a cytoplasmic determinant which in turn is probably always dependent upon a gene for its maintenance

and increase, though the genetic basis is known in one case only. In the other group of varieties, no cytoplasmic factors are detected, each character apparently being determined directly by the genes. Very similar characters (*e.g.*, mating types and antigens) thus show these two different mechanisms of inheritance in the two groups of varieties. It therefore would seem that the nuclear gene in the one group of varieties is the functional equivalent of a gene plus cytoplasmic factor in the other group of varieties. These facts first led to the idea that the cytoplasmic factors had been derived originally from the nuclear genes. No other satisfactory way of explaining this remarkable set of circumstances is even now apparent. It was apparently an error to assume that, because the cytoplasmic factors were originally derived from the nuclear genes, they could reunite with these genes under certain circumstances in the macronucleus. This latter part of the hypothesis certainly has nothing to support it any more. However, the comparison between the two groups of varieties of *P. aurelia* still suggests that the origin of cytoplasmic factors in Paramecium is to be traced ultimately to the nuclear genes.

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## DISCUSSION

LINDEGREN: I would like to repeat Dr. Spiegelman's question concerning the virus-like nature of kappa, because it appears to me that Dr. Sonneborn's proposal to defend the contention that the kappa substance is not a virus does not have a firm foundation. The fact that infection is widespread among Paramecia is not an acceptable argument, since we know many viruses that are widely disseminated; the fact that it affects many characters besides the killer character is not evidence that it is not a virus, since that would also be expected of a virus; the fact that it affects mating type specificity is not surprising, since mating type is merely a bar to homozygous copulation and barring copulation might easily result from infection. So far as I know, these manifold manifestations of the kappa substance have not yet been described in the literature, but even if they exist they do not prove that the substance is not a virus.

These arguments are all very pertinent to those of us interested in the mechanism of gene action, although they may seem trivial to a virologist. Dr. Sonneborn's data have been used by him to develop concepts concerning the mechanism of gene action. If the kappa substance is a virus, which affects cells carrying the *K* gene, and which gives the affected cells an advantage by killing off competitors, it would appear that no direct conclusions concerning the mechanism of gene action could be drawn from a study of the phenomenon. I would like to ask Dr. Sonneborn if he proposes to draw any conclusions concerning the mechanism of gene action or the structure of the gene from this phenomenon.

SONNEBORN: Before attempting to speak about the main points raised by Lindegren, some of his statements about kappa need to be corrected. Kappa does not affect many characters, as he states, but only the killer character and resistance to the killer fluid. Consequently, his remarks about the effect of kappa on mating are incorrect; the mating types are determined by distinct and different cytoplasmic

factors, not by kappa. Finally, Lindegren's statement that "infection is widespread in Paramecium" is a gross understatement of the facts! In certain species (or varieties), it is not merely widespread, it is universal. It is not only universal, but multiple, every individual being "infected" (to use his terminology) with many kinds of "viruses." These corrections will become clearer in the following comments on the main points he raises.

If kappa were the only cytoplasmic factor in *P. aurelia*, the question of whether it is a virus or plasmagene would be more difficult to answer with assurance, although it is, to say the least, remarkable how it reverses the ordinary virus relations. For "infection" here not only produces no harmful effects on the infected individual, but it confers on the "host" the positive advantage of resistance. Still more remarkable, failure to be infected means death to those that live in the vicinity of infected individuals, not because they "catch" the infection but because they have failed to catch it!

The question of the distinction between viruses and plasmagenes has been discussed in detail by Darlington, L'Héritier, and others. Both of these authors stress how difficult it is to make any satisfactory definitions which would distinguish the two. Darlington holds that the main practical distinction is in the normal mode of transmission: if transmitted normally only through the cytoplasm, the agent is to be considered a plasmagene and hereditary; if transmitted normally through infection, it is to be considered a virus and nonhereditary. On this view, there can be no question but that kappa is a plasmagene and not a virus. L'Héritier, in a thorough discussion of the matter, comes to much the same view, though expressed somewhat differently. He holds that the ultimate distinction between virus and plasmagene is in their origin. If the agent is external in origin, it is a virus; if internal, a plasmagene. So far as I am aware, no better means of distinguishing viruses and plasmagenes have been proposed, and the consequence is that in many cases the question becomes an academic one.

With respect to the cytoplasmic factors of Paramecium, however, it seems to me that the question can be answered with more assurance than is possible in many other cases. In certain species (or "varieties") of *P. aurelia*, all characters that have thus far been studied show the same $F_1$ results as in the killer crosses, indicating similar control by cytoplasmic factors. Moreover, when crosses are made between clones differing in *two* characters, the exceptional $F_1$ pairs in which some cytoplasmic exchange has presumably taken place give evidence that different characters are determined by different cytoplasmic factors: the mate that previously lacked both characters may produce a clone that has acquired one, the other, or both of these characters. If the conclusions drawn from these observations continue to be supported by further investigations, the

virus interpretation will certainly be excluded. It would be a *reductio ad absurdum* to postulate that every individual of every race of certain species must be infected with many (possibly thousands) different kinds of viruses in order to develop its normal characters. The argument becomes even more absurd when one notes that in closely related species the same or corresponding characters are determined by the genes directly without the detectable intermediation of cytoplasmic factors. All of these relations are in the literature (*10, 11, 12, 13*). The facts that in these species of *P. aurelia* we seem to be dealing not with a sporadic case, but with an invariable and normal system of determination of characters, and that not a single case of direct control by genes in the absence of an intermediate cytoplasmic factor has been discoverable in this material, seem to me to make the virus interpretation of the cytoplasmic factors of Paramecium improbable. However, if Lindegren wishes to use the term "virus" to designate such a normal and invariable component of the genetic system, that is a matter of personal taste; the important thing is not the names we call things, but the things themselves. As long as the properties and roles of these cytoplasmic components are clearly kept in mind, nothing else really matters. I prefer not to use the term virus for them, for it can only obscure what may turn out to be their most important characteristic—namely, that they constitute a regular and normal feature of the genetic system.

My answer to this first question raised by Lindegren implies my answer to the second. If the cytoplasmic factors of Paramecium are a normal part of the genetic system, then they cannot be ignored in our thinking about the mechanism of gene action. In the paper I have just given, I emphasized more the conclusions I have withdrawn than those I would draw from studies on kappa, because our newer information renders some of my previous notions untenable. As matters now stand, it seems to me that the reproduction of kappa in the cytoplasm is dependent upon a product of gene *K*. Further, no known gene is capable of initiating the production of kappa. Finally, the comparison between different varieties of *P. aurelia* leads me to suspect that the cytoplasmic factors arose originally from the genes. This origin is indicated by the fact that genes alone in certain varieties are equivalent to genes plus cytoplasmic factors in other varieties. The varieties in which cytoplasmic factors are detectable reveal, so far as we can now see, no physical connection between gene and plasmagene; for the study of this, one must turn to those varieties in which the two assumed components of the ancestral gene are still in invariable physical connection. If this view of the matter turns out to be correct, then study of the properties of cytoplasmic factors and their relations to the gene in Paramecium is a study of gene structure and action.

In this connection, the hypothesis proposed yesterday by Spiegelman seems to me difficult, if not impossible, to reconcile with what is known of kappa and gene $K$. Spiegelman postulates that all genes continually produce a corresponding plasmagene. But kappa, which behaves otherwise like a plasmagene, is not currently gene-produced. Nor is it possible to identify kappa with precursor, enzyme, or substrate, the other three components in Spiegelman's scheme. I am, however, inclined to believe that Spiegelman's hypothesis is generally but not invariably applicable. I have stated elsewhere (*11*) my reasons for believing that the relations between cytoplasmic factor and gene in Paramecium may be exceptional because of the unique nuclear dualism (macronucleus and micronucleus) in ciliated Protozoa. The relative inertness of the micronuclei could, as I have stated, underlie the complete dissociation of plasmagene and nuclear gene in this material. It seems to me, therefore, that the special features in Ciliata may provide the exceptions that prove the rule of the Spiegelman hypothesis. I should like to call on Spiegelman to express his views on this matter.

SPIEGELMAN: Before undertaking a direct answer to the question of the relation of kappa to the plasmagene-substrate concept of gene action I should like to say a few words about the "virus" aspect of the kappa problem, about which so much has been said here and which I am afraid I started. In view of the fact that kappa is not genically generated yet requires a proper genome for its propagation in the cell, the comparison with the virus situation is of course inevitable. I raised the question of the virus-like nature of kappa not in any attempt to identify kappa as a virus but simply to emphasize the difficulty if not impossibility of experimentally distinguishing between the two. It is perhaps a great temptation to make the identification, in which case the facts on kappa need not seriously be considered in our speculations about the nature of gene action. Certainly my own particular brain child would have a less difficult time in its struggle for survival if it didn't have to contend with Sonneborn's "killer." Nevertheless, I feel it would be a grave error at the present time to classify kappa as a virus, with all that this would imply as to exogenous origin and unrelatedness to normal physiological and genic processes. This would certainly have the unfortunate result of slamming the door on speculations which could undoubtedly be far more fruitful and suggestive for our understanding of the nature of the gene.

I should certainly agree with Sonneborn that it is difficult to reconcile the results of his ingenious experiments on kappa with the gene mechanism I suggested yesterday. Indeed, it would be impossible if by reconciling we mean directly identifying kappa with one of the explicitly stated components of this scheme. It is not surprising that this situation obtains, since the hypothesis I proposed was specifically formulated to describe and unify the known data on the relations between genetic and enzymatic constitution. The components postulated (plasmagene, precursor, enzyme, and substrate) therefore refer specifically only to this problem. There are, however, implicit properties and relations inherent in the mechanism which permit a possible comparison between it and the kappa case.

We may state the fundamentals of the kappa situation in the following way. There exists a purely cytoplasmic component (kappa) which can maintain itself only in the presence of a gene $K$. Since the latest evidence indicates that kappa is not gene-generated (by $K$ or any other gene) we must conclude from the requirement that $K$ be present that a product of $K$ is necessary for the duplication of kappa. This kind of relationship between a purely cytoplasmic (not gene-generated) component and a gene may be described in terms of the plasmagene hypothesis according to the diagram given below.

$$K \Longrightarrow Pl_K \xrightarrow{+M} 2(Pl_K)$$
$$\Big\updownarrow +\kappa$$
$$(Pl_{K\kappa}) \xrightarrow{+M} 2(Pl_{K\kappa})$$
$$\Big\updownarrow +Pr+S_K$$
$$(Pl_{K\kappa}E_K S_K) \xrightarrow{+M+Pr+S_K} 2(Pl_{K\kappa}E_K S_K)$$
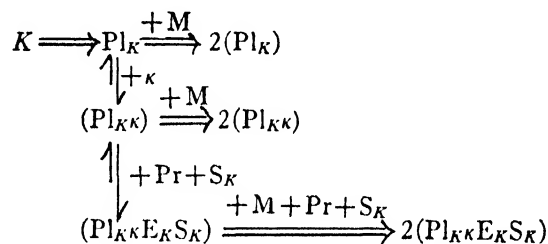
Relation of kappa to the plasmagene mechanism of gene action. Double arrows refer to duplicating processes; M refers to cytoplasmic material. For simplicity, inactivation steps are omitted. See text below for identification of other symbols.

Gene $K$ generates plasmagene $Pl_K$, which in the absence of kappa reproduces nothing but itself. If kappa is available, however, the new plasmagene complex $(Pl_{K\kappa})$ is formed, which duplicates itself thus generating kappa. The complex $(Pl_{K\kappa})$ has the further property, not possessed by $Pl_K$ alone, that it can mediate the production from precursor Pr of the enzyme $E_K$ necessary for the formation of the killer substance from substrate $S_K$. This enzyme-producing capacity possessed by $(Pl_{K\kappa})$ differs in no way from that already described in the plasmagene hypothesis, and need not further be detailed here. It is evident from the data that the self-duplicating capacities of $Pl_K$ and $(Pl_{K\kappa})$ are relatively poor; hence the necessity for having the $K$ gene in the genome to keep replenishing the supply of $Pl_K$. As I have tried to emphasize in my discussion of the plasmagene-substrate hypothesis, a relation of this kind between the relative generating capacities of the gene and its plasmagene is probably the rule. It is interesting

to note that, in complete analogy with the melibiase case, this scheme predicts that it might be possible to maintain kappa in the absence of the gene $K$ by means of large amounts of $S_K$, the substrate transformed into killer substance by $E_K$, if this substance could be identified and obtained.

It is evident from the above that it is formally possible to describe the pertinent facts of the kappa case within the confines of the basic assumptions and concepts of the plasmagene hypothesis.

The assumed relation of kappa to the plasmagene of $K$ in so far as the production of more kappa is concerned is one that would probably obtain in the case of any purely cytoplasmic component (*e.g.*, virus) which required the proper genotype for duplication. If all that kappa did was to multiply in the cytoplasm, there would be relatively little interest in making any comparisons with gene mechanisms. What makes the kappa situation of unique interest to the problem of gene action is that kappa appears to be part of the normal mechanism through which the gene $K$ exerts its particular control over cellular enzymatic constitution. From this point of view it becomes impossible to ignore the results on kappa in consideration of the nature of the gene and the mode of its action. This is even more pointedly emphasized by the existence of other characters whose inheritance involves essentially the same type of mechanism.

Sonneborn points out that the kappa situation is an exceptional one. The far more usual relation between gene and character in the ciliates is one that need not invoke any purely cytoplasmic factor and can be adequately described by the plasmagene-substrate theory of gene action. He correctly emphasizes that the relative rarity of its occurrence does not detract from its interest and indeed may even add to it in view of the fruitfulness usually attending careful analyses of deviations from the norm.

If we take, for the purposes of the present discussion, the plasmagene theory I proposed yesterday as the usual mechanism of gene action, we see from the above diagram that the general pattern of gene-plasmagene-enzyme relationship need not be very different from normal in the kappa case. Except for the assumption that the combination of kappa with $Pl_K$ converts the latter to a functional plasmagene (as far as killer character is concerned), the details and principles of gene action in the kappa and the normal cases are identical.

The most intriguing aspect of the problem centers around the origin of these additional cytoplasmic components, which are necessary for the complete functioning of the corresponding genes and which represent the unique and distinguishing feature of this particular variation of the normal mechanism of gene action. The most satisfying solution to the question of the origin of such units as kappa would obviously be one that would involve their derivation from one of the components of the normal mechanism.

It seems to me that the plasmagene hypothesis itself, and the proposed modification of it to include kappa as a normal unit in its functioning, immediately suggest a quite reasonable hypothesis on how such particles can arise. We may suppose that there existed a gene $K'$, which produced a plasmagene $Pl_{K'}$ that already contained kappa as a component and therefore did not require the addition of kappa to produce either the $E_K$ enzyme or kappa. This would then represent the normal situation of gene-plasmagene-enzyme relationship. In view of the assumed replica relation between a gene and its plasmagene this would imply that kappa was also a component of $K'$. At some time, however, $K'$ changed to $K$ by losing its kappa portion. From that moment on, no more kappa could originate from the nucleus, since all replicas of $K$, whether genic or plasmagenic, would be missing the kappa unit. Although the plasmagene $Pl_K$ originating from gene $K$ presumably cannot form the enzyme $E_K$ the potentiality for synthesizing this enzyme need not be lost to the cell by the change from $K'$ to $K$ as long as some kappa is available in the cytoplasm. This, by the very nature of the plasmagene hypothesis, must have been the case at the moment of the mutation and for some time thereafter, since the original $Pl_{K'}$, plasmagenes which contained kappa existed in the cytoplasm and would be expected to persist for a little while. The final disappearance of the old $Pl_{K'}$ need not result in the disappearance of kappa, since the kappa released by their dissolution could recombine with $Pl_K$ being generated by the mutant gene $K$. This in effect would restore the original self-duplicating system and the cell would thereby retain the power not only to generate more kappa but also to synthesize the enzyme required for the phenotypic expression of the old $K'$.

This interpretation of the origin of such units as kappa represents an interesting mechanism whereby cytoplasmic components can buffer the effects of loss mutations involving parts of genes. It also offers a very simple explanation of why one and the same character would be controlled by the normal mechanism in one variety of protozoa and by the "kappa" mechanism in a closely related one. Only one mutational step need intervene to convert one into the other.

In any case it does offer a point of view that unifies the cytoplasmic phenomena observed in yeast and protozoa in terms of one basic mechanism of gene action. This hypothesis obviously forces us to place the highest importance on the kappa situation, despite its relative rarity, since it leads us back to the idea offered earlier by Sonneborn that at one time kappa was actually part of a gene.

SONNEBORN: Dr. Spiegelman's clever scheme for reconciling the Paramecium facts with the hypothesis of gene action he favors seems to me formally

satisfactory. The implication of the evolutionary origin of kappa from gene $K$ is of course in complete accord with my own conclusions on the matter.

It should be stressed, however, that when a killer heterozygote $(Kk)$ undergoes autogamy, the resulting $kk$-plus-kappa clones normally lose kappa very quickly. The evidence shows that, if there is a required substrate, it is always present under our conditions of culture. Yet Spiegelman's prediction that the assumed $(Pl_K x E_K S_K)$ complex might maintain itself after the substitution of $k$ for $K$ in a killer is certainly not normally fulfilled. This appearance of the recessive trait in cytoplasmic descendants of those that showed the dominant thus seems to weigh against Spiegelman's suggested buffering action of plasmagenes on loss mutations.

EPHRUSSI: Since the question of the relationship between plasmagenes and viruses has just been raised, it might be interesting to bring to the attention of the audience the case of $CO_2$ sensitivity in Drosophila, which has been studied by L'Héritier and which shows some striking analogies with certain observations of Sonneborn.

The $CO_2$ sensitivity was discovered some 12 years ago by L'Héritier and Teissier in the course of experiments on selection in mixed populations. In these experiments $CO_2$ was used as anesthetic, instead of ether. Normal flies undergo immediate narcosis when placed in $CO_2$ and immediately recover their normal activity when returned to air. Flies belonging to an ebony stock, however, were found to behave very differently; after narcosis they appear paralyzed, exhibit few movements, and soon die.

Crosses involving these flies soon showed that the $CO_2$ sensitivity is hereditary but cannot be traced to any gene or chromosome. It is a case of "cytoplasmic inheritance," peculiar only in that there is some transmission through the spermatozoon. This inheritance follows definite and rather complicated empirical rules. In particular: At 20° C. the sensitive stock breeds true and only exceptionally gives a few resistant offspring. Crosses of sensitive females to resistant males give, in the first generations, only sensitive offpring, but some resistant flies always appear in later generations. Crosses between resistant females and sensitive males, on the other hand, give a mixed $F_1$. Sensitive males from such an $F_1$ never transmit the sensitivity if crossed to resistant females, while their sisters again produce a mixture of sensitive and resistant offspring.

In recent years L'Héritier and Sigot have studied the effect of temperature on $CO_2$ sensitivity. They have found that heat treatment (30° C.) can "cure" the sensitive flies and that the cure can be of two types, temporary or permanent. The phenomenon of temporary cure is not yet understood, but the study of permanent cure, which in most cases involves both the soma and the germ line, has led to some very significant results, among which I should like to quote only those important in the present connection. The ease with which the permanent cure is obtained depends first of all on the origin of the treated organism. It is most easily produced in the $F_1$ offspring of crosses of resistant females by sensitive males. The permanent cure of the germ cells is obtained in males only if the heat treatment is applied during embryonic, larval, or pupal development; in females, on the contrary, the cure can be obtained by exposure to heat during the entire life cycle, including the imago. These results suggest that the phenomenon is connected with the active phases of gametogenesis, and the experiments have in fact shown that mature gametes are not affected by the heat treatment.

All these facts have led L'Héritier to an interpretation very analogous to that of Sonneborn; namely, that the effect of high temperature is probably based on a discrepancy between the rate of multiplication of the cells and that of the cytoplasmic units responsible for the $CO_2$ sensitivity.

Recently L'Héritier and Melle Hugon de Scoeux performed a series of transplantation experiments in order to see whether resistant cells can be contaminated by the agent of $CO_2$ sensitivity. It was found that a "resistant ovary" transplanted into a sensitive larva usually yields some sensitive offspring. The implantation of a "sensitive ovary" into a resistant larva results in (a) sensitivity of the imago (host), and (b) some sensitive offspring from the "resistant ovary" of the host. The host can be made sensitive also by implantation of the brain of a sensitive donor. Lastly, it was found that transfusion of lymph (or of a cell-free supernatant of centrifuged lymph) confers sensitivity to a resistant recipient. If the injection is made into a larva, the imago is sensitive on hatching. If it is made into an adult fly, the latter becomes sensitive after about 10 days at 20° C.

It is clear that under these circumstances it is a matter of definition whether we call the agent of $CO_2$ sensitivity a plasmagene or a virus.

SONNEBORN: There can be no doubt that there are many interesting parallels between the work of L'Héritier and his co-workers and the work on Paramecium. Another parallel not mentioned by Ephrussi is that after crosses of $CO_2$-sensitive to $CO_2$-resistant, the cultures of sensitives extracted from the hybrids are regularly less stable than the original sensitive stock. We too find an increase of instability following crosses, and suppose this indicates that other genes or cytoplasmic factors play a role in the maintenace or disappearance of kappa.

On the other hand, there are some differences between the Paramecium and Drosophila investigations. (1) The temperature effect on Drosophila is seemingly due to a differential effect on the rate of

increase of the cells and the rate of increase of the cytoplasmic factor. The temperature effect in Paramecium is a direct destruction of kappa, for all kappa in a killer Paramecium may disappear in the absence of a single fission. (2) In Drosophila there has been found no genic difference related to the control of the cytoplasmic factor. In Paramecium, kappa is dependent upon the gene $K$ and disappears in homozygotes for the allele $k$. (3) The active cytoplasmic agent is transferable from cell to cell without cell fusion in Drosophila. In Paramecium this has thus far proved impossible. The sensitive stock 47 has the genotype ($KK$) appropriate for the maintenance of kappa, and kappa is maintained in it when introduced by cytoplasmic transfer during exceptional conjugations; but stock-47 animals that do not fuse cytoplasmically with killers have never acquired kappa when subjected to fluid in which killers have lived, or to crushed killers.

STERN: Dr. Sonneborn's results recall Jollos's work on *Dauermodifikationen* in Paramecium and Arcella. Similar to the work on enzymatic adaptation in yeasts, Jollos induced new properties in these protozoa and showed that some of these properties were located in the cytoplasm and were reproduced over long periods. The gradual decline in the strength of the newly acquired properties has always suggested a falling behind in their reproductive rate as compared to the animals as wholes, and some of the data, particularly on Arcella, may well be explained by segregation of a small number of extranuclear particles.

SONNEBORN: I have in my paper a discussion of the relation of our work to that of Jollos on Paramecium, though I had to omit this in the oral presentation because of lack of time. As Dr. Stern points out, our new results also suggest explanations of Jollos's work on Arcella, in which old culture fluid appeared to induce *Dauermodifikationen*. With Arcella, as with Paramecium, the characters examined formed a quantitative series, with the normal at one end of the series. Each character of this series was, on the average, persistent for a considerable period during vegetative reproduction. There was also, within limits, a correlation between period of exposure to the inducing conditions and the period of persistence of the *Dauermodifikation*. With respect to these features, therefore, the interpretation I have suggested for Jollos's work on Paramecium would apply also to his work on Arcella. There is, however, one point of difference which could be significant. Jollos never obtained irreversible changes through prolonged application of the inducing conditions. In other words, if changes in concentration of cytoplasmic factors were involved, he never isolated viable lines entirely lacking the factor. This could be due to his methods, which were not well adapted to finding such lines if they occurred with low frequency, or it could, unlike the killer situation, be due to the constant initiation of such factors in low concentration by the genes.

Dr. Stern's suggestion is in a sense the opposite of the one I have made. I have assumed Jollos's inducing treatments lowered the concentration of some previously present cytoplasmic factor in a way comparable to our reduction of kappa concentration by exposure to high temperature. Dr. Stern suggests that Jollos's inducing treatments may have induced the formation of a cytoplasmic factor different from those already present and that the new factor reproduced less rapidly than the cells. This seems to me a less satisfactory possibility, because it would require different mechanisms to account for Jollos's Paramecium and Arcella *Dauermodifikationen*. As the Paramecium *Dauermodifikationen* disappeared when the animals multiplied slowly (during fertilization periods), their loss could scarcely have been due to a slowly multiplying cytoplasmic factor. On the contrary, such observations indicate that the loss was due to the increase in concentration of a factor that had been present in lower concentration during the period of manifestation of the *Dauermodifikation*. I am therefore inclined to interpret the Arcella phenomena in the same way. Obviously, however, what is needed in order to elucidate the phenomena is a repetition of Jollos's work, utilizing the newer methods of analysis.

ALTENBURG: In view of the recent findings just reported by Dr. Sonneborn, it is no longer necessary to assume any direct relation, in point of origin, between $K$ and kappa. One can account for the experimental findings by assuming that $K$ arose first by mutation from $k$, and that kappa later arose by mutation, independently of $K$, from self-reproducing bodies normally present in the cytoplasm. The only action of $K$ might be that of preventing kappa from killing the Paramecium. The gene $k$ might then do either of two things: (1) it might destroy kappa, or (2) it might cause reverse mutation to the nonkiller type of particle. The mutation rate would have to be high in case (2) applied, but an analogous case would be the frequent mutation of chloroplasts from green to white under the influence of the variegated gene in rice and in some other variegated plants.

It might be pointed out here that the number of green symbionts in *Paramecium bursaria* is about 250, and that this is the same as the number of the bodies responsible for the killer effect in *P. aurelia*, as just reported by Sonneborn and Preer. This remarkable correspondence in number suggests the possibility that the bodies in question are related to the green symbionts, having possibly been derived from green symbionts in a common ciliate ancestor. Those in the *P. aurelia* line of descent would then have lost their green color but would still have retained some useful relation to their host (possibly still nutritional, as in the manufacture of vitamins)

and in addition would have acquired new properties (as the killer effect) in certain lines of *P. aurelia*.

SONNEBORN: Dr. Altenburg's suggested interpretation of the action of gene *k* is, of course, identical with Darlington's. I have already pointed out objections to that interpretation. Now that we know approximately the number of kappa particles in a cell, it seems to me more incredible than ever that the transformation of killers into sensitives could be accomplished by mutations of kappa. The mutation of a few hundred particles of kappa per cell within a few cell divisions (1 or 2 days) seems to me little short of a miracle. One must get rid of *all* kappa within a few fissions to account for the failure to maintain kappa when *K* is reintroduced a few fissions later.

As to Dr. Altenburg's interpretation of the action of *K* in preventing kappa from killing the Paramecium, it is known that *KK* animals are sensitive both in the absence of kappa and when kappa is present in low concentration. One must therefore make the totally unsupported assumption that kappa would kill its possessor by a different killing action from the one involved in the killing of sensitives. There is not the slightest indication that kappa is involved in any sort of killing other than the one described in our papers, and, as I have said, this sort is not prevented by the action of K in the absence of kappa.

Without discussing the cogency of the facts or arguments set forth by Altenburg in support of his contention that kappa is descended from symbiotic green algae, two observations seem to me to dispose effectively of this possibility. First, no colorless bodies at all like these green algae can be found in *P. aurelia*. Second, Preer has had under observation the killer *P. bursaria* reported upon by Chen. When first received, this killer *P. bursaria* had typical green algae; eventually, it lost its algae but remained nevertheless a killer.

It seems unnecessary to repeat here the evidence for the origin of kappa from *K*, as this is given in the concluding part of my paper and in the discussion with Lindegren. While this is admittedly speculative, it is based on the facts as known in Paramecium. I can see no evidence in the Paramecium work indicating the origin of kappa from different self-reproducing bodies normally present in the cytoplasm. Moreover, this interpretation, while conceivable, leaves totally unexplained the remarkable difference in genetic systems between the two groups of varieties of *P. aurelia* on which the alternative view is based.

LWOFF: Can sensitives be immunized against the killer fluid by exposure to low concentrations?

SONNEBORN: I tried this experiment some eight years ago, using variety 2 killers, and we have more recently tried cultivating *KK* sensitives that had been exposed to variety 4 killers and had been re-

moved after developing only early stages of the killing reaction. In both cases, the results were negative: the clones developed from the exposed animals showed no change in sensitivity.

LATARJET: What is the rate at which killers become sensitive when the genotype changes from *Kk* to *kk*?

SONNEBORN: The change appears first after about four fissions, though different animals of the same clone change at different rates. Most become sensitives by the eighth fission, though occasionally some animals remain killers somewhat longer. Other experiments, designed to test how long kappa remains in such animals by finding out whether they would remain killers if gene *K* were reintroduced, indicated that kappa disappeared by the fifth fission. Experiments of this sort are too laborious to detect anything but the usual condition. Hence, kappa may rarely persist longer.

There are two important questions involved here. First, does the killer action persist a short time after kappa disappears? On the surface, the data indicate a positive answer, but the possible persistence of killing fluid produced earlier by the animals makes a decision impossible without further investigation. Second, does kappa multiply a little after *K* is replaced by *k*, is it actively destroyed, or is it simply passively segregated in diminishing concentration without increase or destruction? This question also cannot be answered until new, precise determinations are made, taking into account our new knowledge of particle number and washing animals free of the killer fluid at each stage under examination. The persistence of killing action after even four fissions would bring about a reduction of kappa concentration to 1/16 its original level if kappa ceased multiplying as soon as *K* was replaced by *k*, and this concentration should not be adequate for killing action. However, there are complications. The new genotype and the old are both present for a while after fertilization: the old in the fragments of the old macronucleus, and the new in the newly forming macronuclei. There is reason to suspect that the old genotype rules for two or possibly more fissions. The new macronucleus is not fully developed until then, and the fragments of the old macronucleus are still capable of regenerating at this stage. This is perhaps enough to show that the analysis of the details in the transformation of killers into sensitives will not be easy, if it can be done at all.

WHITE: What is the importance of the killer action in nature? Presumably there must be some advantage associated with the sensitive genotype; otherwise sensitives would eventually disappear in nature.

SONNEBORN: The role of the killer action in nature cannot be very well estimated. Our observations are made on small volumes with high concentrations of paramecia, a condition not usual in

nature. The killer fluid probably contains very small amounts of killing substance even under these conditions; moreover, the killer fluid is unstable. Killer action may therefore be weak or practically non-existent in the relatively enormous volumes of ponds and streams with their ordinarily low concentration of paramecia. However, if there is any killer action in nature at all, Dr. White's point seems well taken and one might expect some compensating advantage of sensitives to account for their survival and predominance in nature.

MONOD: Can killer *P. aurelia* kill other species?

SONNEBORN: They do kill other species of Para-mecium but, so far as is now known, they do not kill animals of other genera.

ANDERSON: What is the nature of the killing substance? Will it act at low temperature?

SONNEBORN: We know very little about the nature of the killing substance. It does not dialyse through cellophane. It is destroyed almost immediately at 55° C. It disintegrates within one day or less at room temperature, but can be preserved for several days at 7° C. Killing action proceeds at both low (10°) and high (30°) temperatures, but more rapidly at the latter.

# NUCLEAR AND CYTOPLASMIC FACTORS CONTROLLING ENZYMATIC CONSTITUTION

## S. SPIEGELMAN[1]

Modern physiological research has emphasized the predominant role of enzymes in controlling cellular processes. On the basis of overwhelming evidence we accept as established the fact that a cell can do whatever it does by virtue of the enzymes it contains. Genetics, on the other hand, has provided us with another set of units, genes, which are critical in determining cell properties. It is customary to synthesize the results of these two biological disciplines in terms of a relationship between their fundamental units. Thus, it is commonly assumed that the genotype determines the phenotype by virtue of genic control over enzyme synthesis. On this basis, a mutant would differ from the normal type because the introduction of an atypical gene would result in a modification of the cytoplasmic enzymatic constitution.

The question now naturally arises, what is really meant by such statements as "genes control enzymes" in terms of the mechanism and extent of such control. It is clear that the gene must in some manner influence one or more of the steps in the chain of reactions leading to the transformation of protein into some specific enzyme. Before one could profitably speculate, therefore, on the mechanism of gene-enzyme relationships, it would seem essential to obtain some information on how proteins are transformed into enzymes and what connections such transformations have with other metabolic processes. Some of the basic issues of the problem may be posed in terms of the following questions:

(1) How are enzymes formed in cells?
(2) What mechanisms are available for the maintenance of normal enzymatic constitution?
(3) How far and in what directions can the enzymatic make-up of a cell with a given genome be modified experimentally?
(4) How does this enzymatic variation depend on the genome?

The present paper will attempt to summarize and interpret the results of experiments with adaptive enzymes in yeast designed to obtain information on these and related problems.

## ENZYMATIC ADAPTATION

The phenomenon of enzymatic adaptation may be simply stated in the following terms: a population of cells placed in contact with some substrate acquires, after the lapse of some time, the enzymes necessary to metabolize the added substrate. The

removal of substrate leads to the disappearance of the enzyme system it evoked. So widespread is this phenomenon that it is now customary to follow Karström (6) in designating as "adaptive" those enzymes that are produced as a specific response to the presence of the homologous substrate. Such enzymes are differentiated from the "constitutive" ones, which are presumed to be synthesized by the cell at all times regardless of the presence or absence of their homologous substrates.

It has been noted previously (19, 20) that the reality of the qualitative difference implied by such a division of enzymes into two classes may well be questioned. This point will be returned to later and discussed briefly in the light of some more recent data. For the moment it is sufficient to point out that the existence of the phenomenon of enzymatic adaptation, by allowing for the controlled variation of enzyme content with the aid of substrate, provides a unique opportunity for an experimental analysis of some of the problems raised in the introduction.

Whether or not enzymatic adaptation can be used as a tool for the analysis of the gene-enzyme problem depends on the possibility of performing these enzyme-inducing experiments against a controlled genetic background. The realization of this possibility was afforded only recently through the fundamental work of Winge and Laustsen (35, 36, 37) in Denmark and of the Lindegrens (9, 10, 11) in this country on the genetics and life cycle of the yeasts.

Previous papers (29, 27) have dealt with and defined the conditions necessary for the analysis of the adaptive process against a constant genome. These results emphasized the importance of employing known diploid strains in which the haplophase could be suppressed whenever genetic constancy was desired during the course of an experiment. Haplophase cultures are uncontrollably variable with respect to both biochemical and morphological properties. Such cultures were, however, extremely useful since they permitted the demonstration (28) that capacity to form enzyme in response to substrate is, like other genetically controlled characters, subject to mutational changes. Mutations resulting in both the loss and gain of such characters were observed in haploid clones.

## THE GENETICS OF ENZYMATIC ADAPTABILITY

If a better understanding of the nature and extent of the genic control over enzymatic adaptability was to be attained, it was essential to study the

mechanism of its transmission through crosses of various kinds. Winge and Laustsen (37) were the first to provide information of this nature. They used *S. validus* and *S. mandshuricus,* both of which possess the ability to ferment melibiose. Hybrids were created between these forms and others incapable of fermenting this disaccharide. Their results led them to state that "the presence of a specific enzyme is dominant to its absence in all the instances studied."

These pioneering experiments of Winge and Laustsen were of primary importance in opening up the possibility of a more direct experimental approach to the gene-enzyme problem. Usually genetic experiments study the transmission of such characters as color, shape, or the presence or absence of complete structures. These, of course, involve enzyme activity. However, they usually are the end results of many enzyme-controlled reactions. Any conclusions, drawn from such data, about the nature of the relation between gene and enzyme are necessarily indirect. Here for the first time the transmission of a well-defined enzyme, rather than the products of enzymatic activity, was being analyzed genetically. The necessity for extending these results in several directions was immediately obvious. It seemed desirable to perform experiments which would characterize the phenotype of the two haploids used in the mating, as well as to examine the segregation of the fermentative property in the haploid spores derived from the hybrid. Another point these experiments raised is the following; enzymes are cytoplasmic constituents, and hence the dominance observed could arise from the fact that the enzyme was carried into the cytoplasm of the hybrid as a result of the fusion of the two mating haploids.

Certain features of the mating method employed by Winge and Laustsen made it difficult to analyze some of these questions. It consisted of placing the two spores in contact with each other and allowing the resulting hybrid to grow. The resulting clone was then tested for the desired character. Since the two haploids are consumed in the process, examination of their phenotype is impossible.

The use of the hybridizing procedure developed by Lindegren and Lindegren (10) permitted the further genetic analysis of the inheritance mechanism, In this method, hybrids are produced by mixing haplophase cultures. Since only part of the culture is needed for the mating, the remainder can be used to determine the characteristics of the parent strain. Portions of the clone-culture are also available for back-crossing or mating to other clones of interest.

A genetic analysis of melibiose inheritance was made by Lindegren, Spiegelman, and Lindegren (12), using this hybridization procedure and employing *S. cerevisiae,* which cannot form melibiase, and *S. carlsbergensis,* which can. In all, 175 progenies of the interspecific and of related progenies

were examined. Table 1 gives some typical results. All haploid segregants from *S. carlsbergensis* could form melibiase, whereas all spores derived from *S. cerevisiae* failed to do so, indicating a homozygous condition for the presence and absence of this character in the two strains.

Hybrid I, which was formed by crossing a carlsbergensis haploid with a cerevisiae one, was phenotypically positive, confirming the dominant nature of the fermentative capacity. When asci from such a mating were dissected, three different types were found. Out of 6 asci, from each of which all four

TABLE 1. FERMENTATION OF MELIBIOSE BY PROGENY OF VARIOUS CROSSES BETWEEN S. CEREVISIAE AND S. CARLSBERGENSIS

Four spores were removed from each ascus and designated arbitrarily as A, B, C, D. Plus and minus signs indicate, respectively, capacity to form melibiase and inability to do so.

| Diploid | Phenotype of Copulants | Phenotype of Diploid | Phenotype of Haploid Segregants | | | |
|---------|---|---|---|---|---|---|
| | | | A | B | C | D |
| *S. carlsbergensis* | | + | + | + | + | + |
| *S. cerevisiae* | | − | − | − | − | − |
| Hybrid I | +×− | + | + | + | + | + |
| | | | + | + | + | − |
| | | | + | + | − | − |
| Hybrid IV | +×− | + | + | + | − | − |

spores were recovered and tested, 3 behaved like the original *S. carlsbergensis,* yielding all four segregants as positive; 2 asci yielded 3 positives and 1 negative; and 1 ascus produced the 1:1 ratio of positives to negatives expected of a heterozygote segregating a single dominant gene. Essentially the same results are obtained from any hybrid involving the mating of one of the original carlsbergensis segregants to a negative haploid, the origin of the latter apparently not influencing the results.

When a hybrid (Hybrid IV of Table 1) is formed by mating a positive spore derived as a segregant from Hybrid I with one of the negative haploids from *S. cerevisiae,* the resulting diploid, although phenotypically positive like Hybrid I, always yields a 1:1 ratio of positives to negatives. The peculiar and unexpected distribution of phenotypes observed to occur in Hybrid I may be explained in the following three ways:

(1) Only one gene actually segregates, but the expected 1:1 Mendelian ratio is obscured by mutations of some of the negative spores to positives.

(2) Only one gene actually segregates, but the expected 1:1 Mendelian ratio is obscured by cytoplasmic components originating from the positive spore, which can form the enzyme in the absence of the gene.

(3) Two or more genes, either one of which can mediate the formation of the melibiase, are segregated.

The first explanation seems to be quite definitely ruled out by the fact that no *S. cerevisiae* haploid was ever observed to mutate to melibiose fermentation, even under conditions of long-continued and intensive selection for such mutants. As further evidence against this hypothesis we may cite the 1:1 ratios always observed with hybrids similar to IV, in the segregants of which the same tendency towards such mutations should obtain. Either one of the last two explanations would adequately explain the data. However, as will be shown in the following section, the accumulated evidence strongly supports the cyptoplasmic-factor hypothesis.

Subsequently, similar experiments were done (7, 8) involving another adaptive enzyme, galactozymase. For these experiments, *S. bayanus*, which cannot adapt to ferment galactose, and *S. cerevisiae*, which can, were used. These experiments were complicated by illegitimate diploidization, which made uncertain the origin of sporulating diploids recovered from the mixture of the two haplophases since they need not have resulted from a hybrid mating. Fortunately, another genetic marker was present to mitigate this situation. *S. bayanus* produces large cylindrical cells in both the haplophase and the diplophase, whereas the haploid cells of *S. cerevisiae* are small and round. The hybrid between these two was large and cylindrical, showing that the bayanus type cell is dominant. The segregation of cylindrical versus round cells from such hybrids was regularly Mendelian, thus permitting the distinction between legitimate and illegitimate diploids.

The results of the segregation of the first interspecific hybrid (analogous to Hybrid I of Table 1) again gave asci all of whose spores possessed fermentative capacity. The fact that the same spores which segregated the cell-type character in a perfectly normal Mendelian fashion did not segregate the enzyme served to emphasize the peculiarity in the inheritance of the enzymatic character. Again, as in the case of melibiase inheritance, either the cytoplasmic hypothesis or the multiple-gene hypothesis could explain the data obtained with the legitimate hybrids and their segregants.

### The Extent of Gene Control of Enzyme Formation

In the introductory paragraphs it was pointed out that one of the basic issues of the gene-enzyme problem is the extent of the control exerted by a gene over the enzyme whose synthesis it is presumed to determine. It is clear from the very existence of the phenomenon of enzymatic adaptation that this control is not unlimited. It is evident, for example, that the possession by *S. cerevisiae* of the gene required for the formation of the enzyme galactozymase does not alone guarantee that this enzyme will

be found in the cell. The presence of the specific substrate, galactose, is also required.

The question remains whether intervention by the appropriate gene is necessary every time a molecule of enzyme is formed. In view of the second hypothesis offered for the segregation experiments described in the previous section, the possibility clearly exists that such direct genic intervention need not be necessary.

It was noted in an earlier paper (*19*) that a careful analysis of the kinetics of adaptation could provide data which would be helpful in deciding the relative role of the gene during the formation of an enzyme.

The usual description of gene action assumes that the gene mediates directly the production of the enzyme it controls. From this point of view, every replication of every enzyme molecule would require the intervention of the appropriate gene. On this basis one would ascribe the increase in enzyme in the presence of substrate to the stabilizing influence of the substrate on the enzyme. We may picture this mechanism by the following reaction diagram.

$$
\begin{array}{c}
G_1 \\
\downarrow \\
k \\
P_r \underset{k'}{\overset{}{\rightleftarrows}} E_1
\end{array}
\qquad (1)
$$

Here $P_r$ is the immediate protein precursor, whose transformation yields enzyme, the activity of which is measured. The velocity constant of the transformation from $P_r$ to $E_1$ is $k$, and its magnitude is determined by the gene controlling the reaction. The enzyme $E_1$ is very unstable, however, and reverts to $P_r$ quickly, the velocity constant of the back reaction, $k'$, being very much larger than that of the forward one. Under such conditions only very small amounts of enzyme would accumulate in the cell. We now assume that substrate S stabilizes $E_1$, and that, in the presence of excess substrate, $E_1S$ is formed predominantly. This effectively lowers the value of $k'$. Reaction diagram (1) and the above assumption predict, then, that in the presence of substrate the enzyme activity should increase with time according to the following equation:

$$
E = \bar{P}(1 - e^{-kt}) \qquad (2)
$$

where $\bar{P}$ is the total amount of enzyme finally formed. According to (2), adaptation curves should always be concave to the time axis, and the observed rate of increase in enzyme activity should be greatest at the onset and decrease continuously until maximal activity is reached.

In the course of examining the appearance of various adaptive enzyme systems (galactozymase, maltase, melibiase, hydrogenlyase), well over 1200 adaptation curves have been obtained. In no case does the activity curve resemble the course pre-

dicted by equation (2). In all instances, the initial part of the curve is characterized by a rising rate of enzyme formation. This is then followed by a declining rate portion, when presumably the protein being transformed into enzyme becomes limiting and is finally exhausted. These facts would rule out the simple mechanism suggested by reaction diagram (1).

The increasing rate of enzyme formation with increasing amount of enzyme suggested an obvious modification of the mechanism detailed in diagram (1). Retaining all the properties ascribed to the first mechanism, we add the additional one that the enzyme is part of a cytoplasmic self-duplicating mechanism, such that, once the enzyme is formed, further formation of enzyme molecules can proceed without the intervention of the gene. A mechanism of this sort would result in an autocatalytic transformation of P into E, and would predict that enzyme activity would increase with time according to the following relation:

$$E = \frac{\overline{P}}{1 + e^{a-kt}} \qquad (3)$$

where a is a constant depending on initial conditions and the other symbols have their usual meanings. Table 2 reproduces representative results for two

### TABLE 2. KINETICS OF ADAPTATION

The calculated values were determined by equation (3) on the assumption of an autocatalytic process in which the velocity of enzyme synthesis is a function of the amount of enzyme present.

| Melibiase Activity | | | Galactozymase Activity | | |
|---|---|---|---|---|---|
| Minutes | Calcu-lated | Observed | Minutes | Calcu-lated | Observed |
| 60 | 12 | 8 | 60 | 4 | 2 |
| 90 | 26 | 28 | 90 | 11 | 10 |
| 120 | 56 | 51 | 120 | 26 | 25 |
| 150 | 99 | 98 | 150 | 48 | 48 |
| 180 | 150 | 149 | 180 | 87 | 91 |
| 240 | 218 | 216 | 240 | 152 | 156 |
| 300 | 237 | 238 | 300 | 181 | 181 |

enzymes when the observed values of enzyme activity in an adapting culture are compared with those calculated with the aid of equation (3). There is no doubt that the data lend support to the cytoplasmic self-duplication hypothesis.

### CYTOPLASMIC DUPLICATION OF AN ENZYME IN THE ABSENCE OF THE GENE

An important and critical prediction stemming from the self-duplication mechanism is one that is explicitly stated in the second of the three explanations offered previously for the atypical segregation of enzyme activities; *viz.*, once the process of enzyme synthesis is started it should be able to

proceed in the absence of the gene that initiated it. A test of this prediction is feasible only in a case where the initiating gene can be eliminated. An opportunity of performing this experiment was offered by the existence of such hybrids as IV in Table 1, which regularly segregate the potentiality for enzyme formation. Under such conditions one could be relatively certain that only two out of every four spores carried the gene responsible for the fermentative capacity.

It is evident from the discussion of the phenomenon of adaptation and the role of substrate in it that the ease of exhibiting any self-duplicating cytoplasmic system involved in enzyme synthesis would be greatly facilitated by the presence of substrate. This suggested that a test of this hypothesis could be made by comparing the segregability of fermentative character in the presence and absence of substrate.

Such experiments were carried out by Spiegelman, Lindegren, and Lindegren (30). A hybrid

### TABLE 3. SEGREGATION UNDER NORMAL CONDITIONS OF ABILITY TO FORM MELIBIASE FROM A HYBRID SIMILAR TO HYBRID IV OF TABLE 1

| Ascus Number | Spores | | | |
|---|---|---|---|---|
| | A | B | C | D |
| 8 | + | + | − | − |
| 9 | + | − | + | − |
| 10 | + | − | − | + |
| 11 | + | − | − | + |
| 12 | − | − | + | + |
| 13 | − | + | + | − |
| 14 | + | − | − | + |
| 15 | − | + | − | + |
| 16 | + | − | + | − |
| 17 | − | + | − | + |

### TABLE 4. SEGREGATION IN THE PRESENCE OF MELIBIOSE OF THE ABILITY TO FORM MELIBIASE

| Ascus Number | Spores | | | |
|---|---|---|---|---|
| | A | B | C | D |
| 1 | + | + | + | + |
| 2 | + | + | + | + |
| 3 | + | + | + | + |
| 4 | + | + | + | + |
| 5 | + | + | + | + |
| 6 | + | + | + | + |
| 7 | + | + | − | − |

similar to IV of Table 1 from *S. cerevisiae* × *S. carlsbergensis* was employed. This hybrid was formed in the usual manner and allowed to sporulate, the asci dissected and tested for the character. The results are shown in Table 3. It is clear that the regular 1:1 Mendelian ratio is obtained, characteristic of a heterozygote segregating a dominant gene. It should be noted for later reference that, in handling asci 8 and 9, the agar in which the dissected spores were planted contained melibiose.

In these experiments, the cells came into contact with melibiose for the first time in the test for adaptability after segregation had already taken place. Using exactly the same haploids, the same cross was carried out in the presence of melibiose. Segregations were also allowed to occur in the presence of this sugar. The data on the phenotypes of the haploid segregants obtained in this manner are summarized in Table 4. With the exception of ascus 7, identical heterozygotes treated with melibiose yielded four adaptable spores from each ascus.

The segregation results obtained in the absence of substrate (Table 3) prove that only 2 spores from each tetrad in asci 1-6, inclusive, of Table 4 contain the specific gene responsible for the initiation of the adaptation towards the fermentation of melibiose. Despite this, all four spores from these tetrads produced haplophase cultures which fermented melibiose. It would thus appear that with the aid of substrate we were successful here in obscuring what is normally a simple Mendelian segregation and duplicated the abnormal segregations earlier noted with the interspecific hybrid between carlsbergensis and cerevisiae.

It might be argued that, since all steps were carried out in the presence of melibiose, selection of adaptable mutants from haploids originally unable to ferment melibiose may have occurred. Several specific facts would appear to rule out this possibility: (1) during the testing of many haploid segregants from S. cerevisiae, all of which are negative, no mutation to an adaptable type has ever been observed, whether melibiose is present or not; (2) the same is true of negative haploids derived from heterozygous hybrids; and (3) asci 8 and 9, whose segregants were planted on melibiose, yielded the standard 1:1 ratio.

We are thus led to suppose that the cultures from two spores of each tetrad from the first 6 asci in Table 4 lacked the gene and were able to ferment melibiose only owing to the enzyme-forming factors present in the cytoplasm. If this is correct, it might be expected that removal of the melibiose would lead not only to the disappearance of the melibiase but also, if the cytoplasmic factors were sufficiently labile, to the eventual loss of readaptability in those two out of every four spores of asci 1-6 that lacked the gene.

Such experiments were performed, and, to exclude the complications of mutation away from adaptability, nondividing cultures, suspended in M/15 $KH_2PO_4$ containing glucose, were used. Portions of all 24 adapted cultures originating from the first 6 asci were treated in this way in the absence of melibiose. In varying periods of time, ranging from 7 to 20 days, all these suspensions lost the ability to evolve significant amounts of $CO_2$ anaerobically from melibiose. Twenty-four hours after a suspension showed no significant traces of enzyme activity, a sample was removed and incubated with melibiose

aerobically to test for readaptability. At the same time its ability to ferment glucose was also examined. This was done to avoid testing cells whose physiological condition was seriously impaired by the long and vigorous shaking in a relatively unfavorable environment. With cells unable to ferment glucose, the inability to ferment melibiose would be difficult to interpret. Three suspensions of the original 24 were eliminated on this basis. The data on the asci all four of whose clones survived the treatment are given in Table 5. The removal of the meli-

TABLE 5. READAPTABILITY OF SPORES OBTAINED BY MATINGS IN THE PRESENCE OF MELIBIOSE AFTER HAVING LOST ALL MELIBIASE ACTIVITY

| Ascus Number | Spores | | | |
|---|---|---|---|---|
|  | A | B | C | D |
| 1 | + | − | + | − |
| 2 | − | − | + | + |
| 4 | + | + | − | − |
| 6 | + | + | − | − |

biose leads to the disappearance of the cytoplasmic factors responsible for the appearance of the enzymatic character in all four segregants and the reappearance of the expected Mendelian ratio of 1:1 on readaptation.

This appearance of enzyme in the cytoplasm of cells apparently not carrying the gene could have been due to a passive transfer and subsequent retention of the enzyme which was induced by substrate in the cytoplasm of the parent diploid. That this was not the case was shown by experiments in which portions of all clones were allowed to fall to low enzyme-activity values ($Q^N_{CO_2}$ between 1.8 and 10.1) in the absence of melibiose. Aliquots were then removed and incubated with melibiose, and evidence for regeneration of activity followed at intervals. In all cases, including those that eventually lost their ability to adapt and therefore presumably lacked the gene, marked increases in activity were observed. Activity values greater than 100 were attained in every instance. Furthermore, all the clones were carried in standard media with melibiose and were tested at weekly intervals. At the end of three months they could all without exception ferment melibiose at rates equal to or greater than the ones they started with. This period is equivalent to over 2000 cell generations. It is evident from these results that the enzyme is actually synthesized in the cytoplasm of these cells in the absence of the genes.

In view of these experiments, the atypical segregation values of the interspecific hybrid, and the kinetics of the adaptive process, it seems difficult to avoid adopting the cytoplasmic hypothesis. All three types of results can easily be understood in these terms.

We may therefore explain the effect of melibiose on the inheritance of the corresponding enzyme as follows: because the mating is performed in the presence of melibiose, the cytoplasm of the haploid carrying the gene is packed with melibiase. Since both copulating haploids contribute cytoplasm equally to the diploid, it starts out with some enzyme and builds up more, since it has the gene also. Because sporulation occurs in the presence of melibiose and because the sporulation period is characterized by growth and considerable storage, the enzyme molecules are stabilized and possibly increased in amount. Each of the four haploid segregants derives its cytoplasm from the diploid hybrid, and it follows that each will have enzyme molecules in its cytoplasm no matter what its genetic constitution chances to be. Finally the enzyme molecules are stabilized and are duplicated in the cytoplasm of the clones derived from the spores which did not carry the gene as long as they are kept in contact with substrate.

The fact that Hybrid IV normally gives typical Mendelian segregation of the capacity to form enzyme, whereas Hybrid I does not, may be explained by the fact that the carlsbergensis cytoplasm has been twice diluted with cerevisiae cytoplasm. The cytoplasmic factors that obscure the Mendelian picture of Hybrid I have thus apparently been diluted in Hybrid IV to the point where they can no longer play critical roles in determining potentiality for melibiase formation.

The cytoplasmic hypothesis received further support from subsequent experiments (7, 8) involving galactozymase. Results analogous to those just described with melibiase were obtained with the *S. cerevisiae* × *S. bayanus* pedigree. Some asci which yielded four adaptable spores were shown to be heterozygous for the gene required to initiate galactozymase production, since on growing the four segregant clones in the absence of substrate two of them irreversibly lost the capacity to adapt to galactose.

Another series of experiments offers indirect support for the importance of cytoplasmic factors in the transmission of enzymatic adaptability, by making the polygenic hypothesis offered as an alternative seem very unlikely. In these experiments, the same *S. carlsbergensis* haplophase employed previously was crossed with a haplophase strain lacking the ability to adapt to either galactose or melibiose. Most of the asci obtained from this cross yielded a 1:1 ratio for the capacity to form galactozymase as well as a 1:1 ratio for the ability to form melibiase, and, furthermore, both characters segregated completely independently. However, a few of the asci obtained produced four spores, all of which exhibited the capacity to form *both* galactozymase and melibiase. This latter result would be difficult to understand on a genic basis but would be expected if in such asci the proper Mendelian

ratio for the two characters was being obscured by the transfer of cytoplasmic components.

The general picture which thus emerges of enzyme formation and the relation of the gene to this process may be described as follows: enzymes may be produced by cytoplasmic components which possess the capacity for self-duplication. In such instances the sole function assignable to the gene is the initiation of the enzyme synthesis. The initiation could be effected by virtue of a low but ever-present capacity of the gene to mediate the production of a few of the cytoplasmic self-duplicating units involved in the formation of the enzyme. The presence of the gene would thus retain indefinitely for the cell the capacity to adapt by forming a large number of enzyme molecules when it comes in contact with the proper substrate.

The introduction of a cytoplasmic component as critically determining in enzyme formation naturally raises a whole host of new problems. Of primary interest are the following two questions:

(1) What is the nature of this cytoplasmic component and what relation does it have to the homologous gene and enzyme?

(2) How does it intervene in the formation of the enzyme?

Actually when the findings on melibiase were reported (*30, 19*) it was suggested that the enzyme itself was the self-duplicating system. Subsequently, Lindegren (7) identified the enzyme with the self-duplicating cytoplasmic unit which he called the "cytogene." It is clear, however, that the evidence thus far presented does not permit a decisive conclusion as to whether the cytoplasmic self-duplicating unit is the enzyme itself or something mediating its formation.

It soon became apparent that an extension of these results in an attempt to answer questions regarding the identity of this cytoplasmic unit and its role in enzyme formation must be preceded by the acquisition of some insight into the physiological and biochemical details of the process. Without further information of this kind on the pathway or mechanism of enzyme formation it seemed impossible to think fruitfully about these aspects of the problem.

An adequate description of the mechanism of enzyme formation must necessarily provide answers to the following questions, among others:

(1) What is the relation of enzyme formation to over-all cellular metabolism?

(2) What is the source of protein for the formation of an enzyme?

(3) What is the source of energy required for its synthesis?

## THE RELATION OF ENZYME FORMATION TO CELLULAR METABOLISM

Earlier workers had already established the fact that enzymatic adaptation requires physiologically

functioning cells. Attempts to obtain adaptation with nonviable cells, or with cells whose physiology has been seriously interfered with by various reagents, have uniformly met with failure. The sole exception was the report by Abderhalden (1) that he had obtained adaptation to galactose fermentation with dried dead yeast cells. This experiment has never been successfully repeated, and his failure to check the possibility that the adaptation may have been due to the growth of a few surviving cells throws grave doubt on the validity of his conclusions. Von Euler and Nilsson (34) reported that adaptation to galactose would not occur when the cells were suspended in ordinary phosphate solution containing galactose, and they maintained that the addition of "Z" factor was necessary. These observations were in direct contradiction to the earlier experiments of Dienert (5), who observed adaptation with washed cells suspended in ordinary phosphate.

Our own experience agrees with that of Dienert. All our adaptations were carried out with thoroughly washed cells suspended in phosphate solutions of substrate. These experiments establish the important fact that enzymes can form in yeast cells in the absence of an external source of nitrogen. It must be noted, however, that the maximal level of enzymatic activity reached under such conditions is about half or less that attained when the adaptation is carried out in the presence of an exogenous source of nitrogen.

Yeasts are cells which possess both an aerobic and an anaerobic metabolic pathway, either of which can be used for purposes of growth. The first problem that was experimentally analyzed was to see whether both of these pathways could be utilized for enzyme formation. Evidence on this question was conflicting. Stephenson and Yudkin (32) state that oxygen has no influence on the adaptive process. On the other hand, Schultz, Atkin, and Frey (17), who also investigated the adaptation of yeast to galactose fermentation, reported a very striking effect of oxygen. This question was reinvestigated (20) with the aid of five known diploid strains. Two of these strains were unable to initiate the formation of galactozymase if they experienced only anaerobic contact with galactose, while the others could form the enzyme anaerobically. However, the rate of anaerobic adaptation in these latter cases was only 1/40 of that attained in the presence of oxygen, and furthermore, this anaerobic adaptability could easily be abolished by dissimilation for a few hours. This same relative inefficiency of anaerobic incubation with substrate in forming the homologous enzyme was also observed with maltose and melibiose.

It might be supposed that anaerobic inadaptability could be explained by the inability of the cell to use anaerobic metabolism for enzyme synthesis. This was tested with one of the strains, which was completely incapable of anaerobic adaptation. The changes in enzyme activity under anaerobic conditions were followed subsequent to periods of aerobic incubation with galactose. It was found that if sufficient aerobic contact with the substrate was allowed, so as to attain an enzyme activity equal to or greater than a $Q^N_{CO_2}$ of 15, further enzyme was formed during a subsequent period of anaerobic contact with the substrate.

These experiments made it quite evident that the anaerobic metabolic pathway could also be used for enzyme formation. The condition that apparently had to be met here was that sufficient enzyme be formed first aerobically so that the cell would be in a position to utilize the energy content of the galactose molecule anaerobically at a rate adequate for the synthesis of more enzyme. We thus have here the interesting biological situation that the substrate not only stimulates the formation of the enzyme but in addition acts as the ultimate source of energy for the synthesis.

The critical role of an energy supply from the metabolic cycle for the adaptive process is illustrated by several other facts. It has been shown (31) that for these strains, in common with others (see 33), the endogenous reserves are not fermentable and can only be used in the presence of oxygen. Such cells, when placed under anaerobic conditions in the absence of a fermentable substrate, do not metabolize. It is therefore not surprising that the capacity to form enzymes anaerobically is so poor.

The same viewpoint suggests that aerobic adaptation occurred with such ease because with oxygen present the cell could draw on the energy coming from the oxidation of the endogenous reserves for the synthetic activity. To confirm this, experiments were performed (19) to examine adaptation times (time required to reach a $Q^N_{CO_2}$ value of 100) when galactose was added at different levels of the endogenous respiration. It was found that little difference in adaptation times was encountered as long as the galactose was added during the early period of the endogenous material before the declining-rate phase was attained. Subsequent to this, adaptation times increased sharply. The surprising fact to emerge, however, was that adaptation, although delayed, still took place when the galactose was added after all metabolic activity had ceased as measured by oxygen uptake and $CO_2$ output. These experiments seem to indicate that adaptation can occur after all the oxidizable reserves have been exhausted and there is no apparent source of energy. This puzzling situation was clarified, however, by the finding (21) that the galactose itself is oxidized by some enzyme system other than the fermentative one, which forms later under its stimulation.

It is evident from these experiments that the presence of a functional aerobic or anaerobic metabolic system is necessary for the formation of the enzyme.

## The Source of Protein for Enzyme Formation and the Interconvertibility of Enzymes

It has already been noted that the presence of an exogenous source of nitrogen raises the maximal attainable level of enzyme activity. This seemed to indicate that the cell could use externally supplied nitrogen for the purpose of increasing its activity. However, the fact that enzyme activity can appear in a nitrogen-free environment implies that there exist endogenous sources of protein, which are available for transformation into enzyme protein. It has generally been supposed that most cells contain very little "storage protein" and that at any given moment most of the protein is functional, enzymatic or otherwise. There existed the possibility, therefore, that when a cell is forced to form a new enzyme it may draw upon existent enzymes as a source of protein. Presumably an interaction of this kind would be most pronounced between unstabilized labile enzymes—i.e., between members of the so-called adaptive group of enzymes.

This possibility was tested (23) in the following manner: one group of cells was fully adapted to galactose and another to maltose. Then a comparison was made of the ability of galactose-adapted cells to form maltase when incubated with an equal mixture of galactose and maltose in the presence and absence of an external source of nitrogen. A similar comparison was made of the ability of maltose-adapted cells to form galactozymase. If such an interaction between enzymes of a cell exists,

### TABLE 6. Competitive Interaction between Adaptive Enzymes

| Enzyme | Maximal Activities ($Q^N_{CO_2}$) | | | |
|---|---|---|---|---|
| | Without External Nitrogen | | With External Nitrogen | |
| | Cells adapted to galactose | Cells adapted to maltose | Cells adapted to galactose | Cells adapted to maltose |
| Galactozymase | 163 | 101 | 232 | 227 |
| Maltase | 86 | 182 | 229 | 248 |

inducing to maximal activity one enzyme of a cell and stabilizing it with its substrate should influence the ability of another enzyme to establish itself in the same cell. The results of a typical experiment, which are given in Table 6, leave no doubt that such an interaction exists. The presence in a cell of maltase depresses considerably the attainable level of galactozymase, and vice versa. Both inhibitions are effectively abolished by the addition of a nitrogen source, which apparently permits the cell to form new enzyme without drawing upon existent proteins.

Another way of studying this phenomenon of interaction is to actually follow what is happening

to the existent enzymes of a cell while a new enzyme is being formed. Thus if one starts out with a culture fully adapted to maltose ($Q^N_{CO_2} = 240$) and adapts to galactose one finds that as the galactozymase activity increases there is a sharp drop in maltase activity so that within four hours practically all of it is gone.

A test of the generality of this type of interaction between cytoplasmic enzymes was made by seeing whether the induction of an adaptive enzyme resulted in any loss of activity in a constitutive one. For this purpose a culture was adapted to galactose,
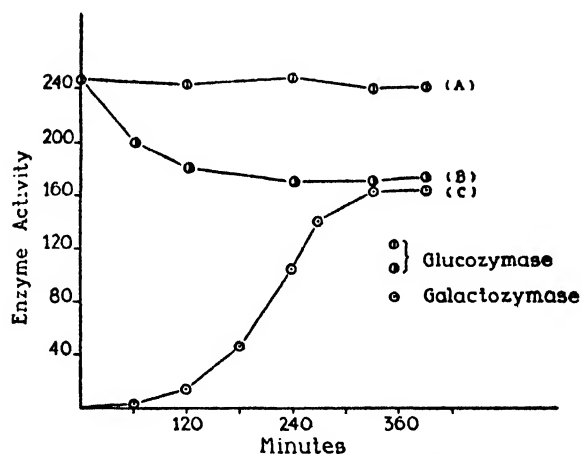


Fig. 1. Interaction between galactozymase and glucozymase during adaptation to galactose in the absence of an exogenous source of nitrogen. Curve (A) is a control, demonstrating stability of glucozymase in a nonadapting culture.

and at intervals samples were removed for simultaneous determinations of glucozymase and galactozymase activities. The results of an experiment of this type are depicted in Fig. 1. The same results are obtained here as in the case of interaction between two adaptive enzymes. To ascertain whether a question of nitrogen supply is involved here also, a control experiment was run in which the adaptation was carried out in the presence of nitrogen. Fig. 3 gives the results. It will be noted that the adaptation rate is increased and the maximal enzyme activity level attained is greater; and, even more significantly, parallel measurements in glucozymase activity during the course of the adaptation show no change.

Thus these experiments provide some information on the question of the protein source during adaptation. If an external source of nitrogen is present this will be employed. In its absence, however, a cell will use existent cellular enzymes as a source of protein to form the enzyme being induced by substrate.

The fact that a constitutive enzyme was observed to disappear during the formation of an adaptive one throws serious doubt on the basis for

the distinction made in the literature on enzyme variation between the two types of enzymes. It emphasizes once more that the only difference that exists is in degree of stability and utilization. The importance of these findings resides in the fact that
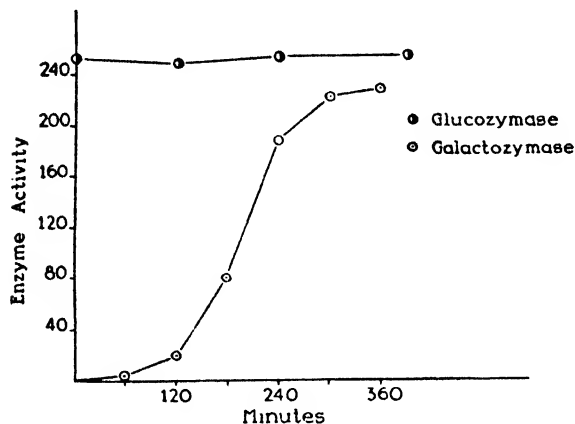


FIG. 2. Protective action of exogenous nitrogen source on glucozymase activity of an adapting culture.
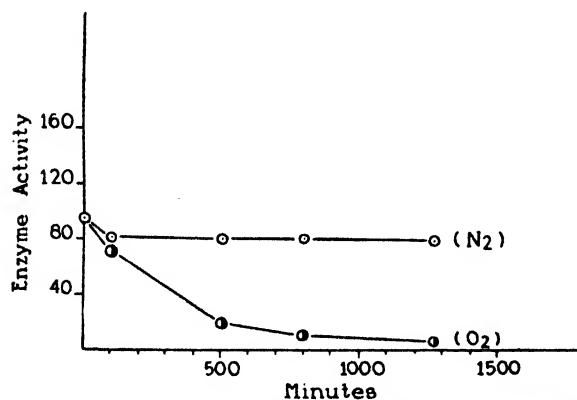


FIG. 3. Comparison of galactozymase stability in absence of substrate under aerobic and anaerobic conditions.

they naturally make it possible to extend the results obtained with adaptive enzymes to enzyme synthesis and maintenance in general.

### THE RELATION OF ENZYME STABILITY TO THE SYNTHESIS OF OTHER ENZYMES .

The finding that the formation of a new enzyme by a cell affects other enzymes suggested that the whole question of enzyme stability and maintenance must be looked at from a different point of view. Whether a particular enzyme survives in the cytoplasm during the course of an enzymatic change would, from this point of view, depend not only on its inherent instability but perhaps even more significantly on the ability of the units involved in its synthesis to compete with other such units for proteins.

An obvious consequence of this is that the stability of a particular enzyme at any given time would depend on the rate of enzyme turnover at that moment. Complete stabilization of enzymatic constitution could thus conceivably be attained if all metabolic activity could be stopped. This conclusion was tested with galactozymase, since it was felt that if this was true for an adaptive enzyme, the instability of which is well known, its generality would be more certain. It will be recalled that, under anaerobic conditions, no detectable metabolic activity can be observed in yeast suspension in the absence of a fermentable substrate. The experiment was therefore performed of testing the stability of galactozymase in oxygen and in nitrogen in the absence of substrate. The results are given in Fig. 3. Except for a slight drop at the beginning, complete stabilization of the enzyme was attained in the absence of metabolic activity. The same suspension in the same period of time lost almost all of its activity when allowed to metabolize its endogenous reserves.

In Fig. 4, the ability of a suspension to retain its galactozymase activity is compared during
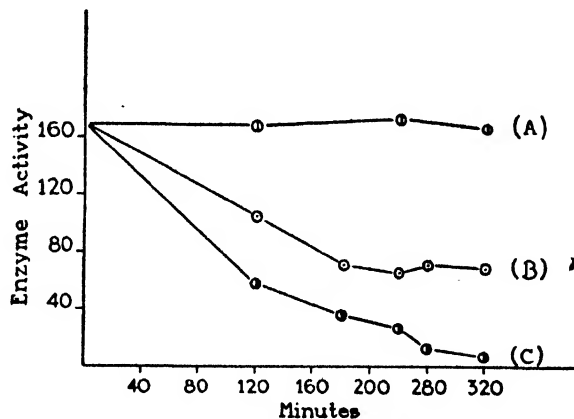


FIG. 4. Comparison of galactozymase stability in cells metabolizing (A) galactose, (B) endogenous reserves, (C) glucose.

endogenous and exogenous metabolism. It is seen that galactozymase is lost faster in the suspension metabolizing externally placed glucose. A control was run in which the same suspension was allowed to consume galactose. No enzyme activity was lost, indicating that substrate can effectively prevent such losses. Since exogenous metabolism of glucose is between two and three times as high as the maximal rate of endogenous metabolism, it is evident that these findings are consistent with the idea that the level of metabolic activity can affect enzyme stability.

Monod (14) in a series of extremely interesting and suggestive experiments on his "diauxie" phenomenon demonstrated a similar inhibitive action of a constitutive enzyme on a variety of adaptive sys-

tems. The fact that the same general conclusions were arrived at gains greater interest in view of the fact that this author used bacteria as his material and employed mainly growth rates as a measure of adaptation.

These results strongly support the concept that enzyme formation and disappearance are closely linked. One would predict from this that any inhibitor which could interfere with enzyme synthesis would freeze the existent enzymatic constitution independently of what substrate was being metabolized. The verification of this prediction will be presented in the next section. The experiment on stability in the absence of metabolic activity would suggest that the inability of a cell to maintain a particular enzyme while metabolizing another substrate is probably not due primarily to an "inherent instability" in this enzyme but rather to competitive interaction between enzyme-forming systems.

### THE INHIBITION OF ENZYME FORMATION

It has been pointed out previously that any procedure that seriously interferes with cell viability or prevents the cell from metabolizing results in a loss of enzymatic adaptability. This only tells us that adaptation, like other cellular synthetic processes, requires a properly functioning metabolic cycle to supply the energy for the proper reactions. It tells us nothing of the nature of the linkage between the adaptive process and the metabolic reactions.

One method of approaching this problem was to attempt to dissociate the process of adaptation from the over-all metabolic cycle. In such cells, where enzyme formation is prevented without affecting the over-all metabolic rate, one could hope to analyze the nature of this link. Dissociations of this kind between synthetic processes and metabolism have been accomplished with the aid of various compounds. Thus the ability of $NaN_3$ to prevent the utilization of metabolic energy for synthesis is quite general, having been demonstrated for such diverse processes as cell division, embryonic development, regeneration, and carbohydrate and ammonia assimilation. It was of some interest to determine whether enzymatic adaptation behaved in a manner similar to these synthetic processes with respect to inhibition by azide.

Since azide is a powerful inhibitor of aerobic metabolism, it was trivial to find that it inhibited aerobic formation of enzyme. Putting azide into the adapting medium is equivalent to establishing anaerobiosis, and we have already noted that anaerobic conditions, if instituted from the beginning, are effective inhibitors of enzyme formation. Of greater interest were the experiments (22) in which the effect of azide was tested on anaerobic synthesis of enzyme subsequent to a period of aerobic incubation with substrate. Fig. 5 describes such an experiment. Cells were incubated aerobically with galac-

tose until the galactozymase activity reached a $Q^N_{CO_2}$ value of 40, after which anaerobiosis was instituted and incubation with the substrate continued. Control suspensions continued to increase their enzyme content in a manner depicted by the first three points and the dashed part of the curve. If at any point in the development of this enzyme activity azide is introduced in concentration of $5 \times 10^{-3}$ M or higher, no further enzyme is formed
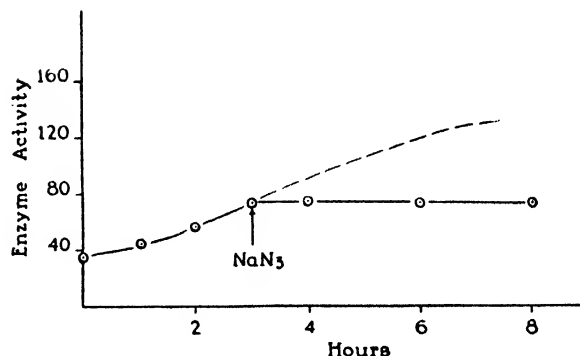


FIG. 5. Effect of $NaN_3$ ($5 \times 10^{-3}$ M) on anaerobic formation of galactozymase.

and the suspension continues to ferment the galactose at the rate attained at the time of the addition. Azide is also effective in preventing cells from utilizing the energy of other fermentable substrates for adaptive purposes. Schultz, Atkin, and Frey (17) have shown that adaptation to maltose fermentation can occur in the absence of oxygen providing a small amount of glucose is furnished. This utilization of the energy obtained from the glucose to form maltase is completely prevented by azide (unpublished experiments). It is also of interest to recall that assimilation of ammonia by yeast cells was shown by Winzler, Burk, and du Vigneaud (39) to be prevented by the same concentrations of azide.

Enzymatic adaptation thus appears to behave in a manner similar to other synthetic processes in that it is possible by the addition of azide to prevent its occurrence without affecting the measured over-all metabolism. It was pointed out previously that the ability of a cell to maintain a particular enzyme is probably closely linked with the intensity with which other enzymes are being synthesized. If azide can effectively prevent enzyme synthesis in general, it follows that this compound should prevent not only the synthesis of some enzyme, but also its disappearance, no matter what substrate the cell was metabolizing nor how fast it was doing this. Fig. 6 gives the results of an experiment testing this possibility. A galactose-adapted culture was allowed to consume glucose at maximal rate under anaerobic conditions. As shown by the broken line, the galactozymase activity disappears quite rapidly.

If at any time in this process, however, azide is added, further disappearance of this enzyme ceases immediately and the enzyme activity remains indefinitely at the level reached when the azide was added. The fact that the same compounds or conditions that inhibit enzyme formation also inhibit
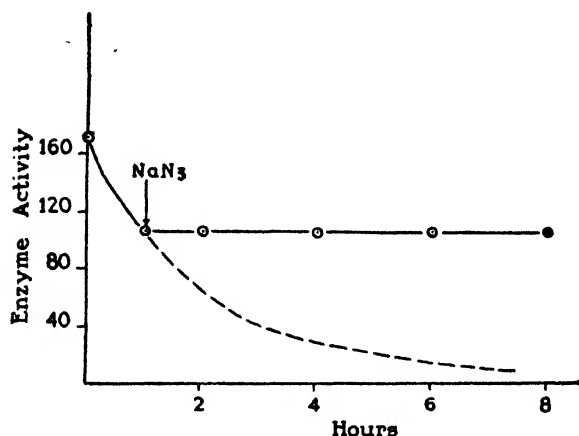


FIG. 6. Complete stabilization by NaN₃ (5 × 10⁻³ M) of galactozymase in cells metabolizing glucose.

enzyme loss makes it difficult to avoid accepting competitive interaction between cytoplasmic enzyme-synthesizing units as playing a critical role in determining cellular enzymatic constitution.

### The Mechanism of Azide Inhibition of Enzyme Synthesis and Its Relation to Phosphorylation

The ability of azide to prevent the formation of enzyme without disturbing over-all metabolism obviously represented a system the analysis of which could provide a clue to the link between the metabolic energy cycle and the synthesis of enzymes. An attempt was therefore made to determine the mechanism of this inhibition.

On the assumption that P-bond energy as generated by the glycolytic system forms the primary source of energy for cell function and growth, experiments were undertaken (26) to examine the effect of NaN₃ on phosphorus metabolism, using radioactive phosphorus (P³²) as a tracer. All the experiments were done under anaerobic conditions. The procedure in these experiments was to suspend cells in an inorganic phosphate medium containing a known amount of tracer P and allow the cells to ferment glucose anaerobically, removing samples at intervals for radioactive and chemical analysis. The results of a typical experiment of this kind are given in Fig. 7. In this experiment 2 × 10⁻³ M NaN₃ was used. It is seen from the upper curve that the presence of this amount of azide did not disturb the ability of the suspension to metabolize, since both consumed glucose at precisely the same

rate. However, whereas the control exhibited a relatively rapid uptake and exchange of inorganic phosphate, the experimental did not. Chemical analyses for total, inorganic, and organic phosphates, and radioactive examination of these fractions, confirmed these results. Azide is able to prevent the
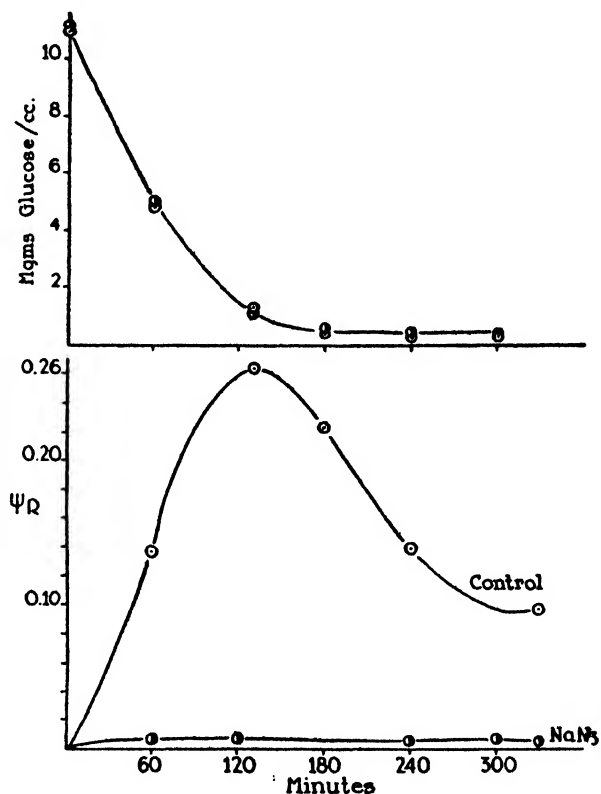


FIG. 7. Effect of NaN₃ (2.5 × 10⁻³ M) on P turnover during anaerobic glycolysis. Ordinate of lower curve represents ratio of specific activity of P inside the cell to that of the P outside. Upper curves describe consumption of glucose in the absence of NaN₃ (open circles) and in the presence of NaN₃ (2.5 × 10⁻³ M) (half-shaded circles).

accumulation and formation of organic phosphate bonds which normally accompany the metabolism of carbohydrates.

These results suggest that the capacity of NaN₃ to prevent cellular utilization of metabolic energy for enzyme synthesis (as well as other synthetic processes) resides in its ability to dissociate carbohydrate metabolism from the generation of energy-rich organic phosphate bonds.

Further experiments were performed (24) with the purpose of determining the site of the uncoupling of phosphorylation from carbohydrate metabolism in the presence of azide. According to the classical glycolytic mechanism, the formation of organic phosphate bonds from inorganic phosphate occurs by the entrance of inorganic P into

the carbohydrate cycle in two places: (1) phosphorolysis of glycogen; (2) the coupled oxidation and phosphorylation of glyceraldehyde phosphate to di-phosphoglyceric acid.

The involvement of the first step seemed unlikely, since no polysaccharide is synthesized by yeast cells in the presence of azide (38). Since it seemed most probable that the azide was affecting the second step, experiments were devised to examine the behavior of the glycolytic cycle at this level in the presence of azide. It is well known that iodoacetic acid (IAA) is a strong inhibitor of the enzyme (triose-phosphate oxidase) controlling this step. It was reasoned that if azide modified the phosphorylative mechanism at this step it might be expected that a parallel change in the sensitivity of the fermentation to IAA might appear, and such was actually found to be the case. A concentration of IAA ($2 \times 10^{-4}$ M) which was sufficient to inhibit fermentation completely within 10 minutes in the absence of azide took 180 minutes to bring the rate down to zero in the presence of $5 \times 10^{-3}$ M $NaN_3$, and failed to affect the rate at all for the first 70 minutes. Lower concentrations of azide protected against IAA inhibition to a lesser extent. Higher concentrations continued to lengthen the period of protection until inhibitory concentrations of azide were reached, whereupon the protective action against IAA disappeared. It should also be noted that azide is completely unable to protect fermentation against the inhibitory action of fluoride, which poisons an enzyme controlling an entirely different step.

## NUCLEOPROTEIN AS A CONTROLLING ELEMENT IN ENZYME SYNTHESIS

Any attempt at elucidating the mechanism of a biological synthesis must of necessity concern itself with the problem of the immediate donor of energy and substrate in the synthetic reaction. The experiments thus far described on the relation between phosphate metabolism and enzyme adaptation have dealt with the formation of organic phosphate bonds. They demonstrated that inhibition of the generation of these bonds is always accompanied by an inhibition of enzyme formation. This tells us only that organic phosphate bonds are required. It does not tell us which particular ones are critical nor does it tell us how they are used. The insufficiency of this knowledge is pointedly emphasized by experiments with dinitrophenol, which, as was independently shown by Monod (15), is also capable of preventing enzyme adaptation without inhibiting over-all oxidative metabolism. However, this compound does not interfere with organic phosphate esterification nearly so effectively as azide. Suppression of only 20% is obtained with dinitrophenol concentrations capable of preventing enzyme adaptation.

Clearly some information had to be obtained on

what happened to the various organic phosphate compounds while a protein or enzyme was being synthesized. Experiments were designed (25) to obtain such information, again using radioactive phosphate. In these experiments the phosphate in the various fractions of the cells was tagged with tracer before the beginning of the experiment so that subsequent movement of the P from one fraction to another could be followed.

The most consistent correlation between phosphorus metabolism and protein or enzyme formation was found in the flow of phosphate *from* the nucleoprotein fraction (NP). This latter is the residue phosphate remaining after successive extractions with water, cold trichloroacetic acid, alcohol, and hot alcohol-ether (3:1).

The behavior of the phosphate in this fraction was followed under various conditions, employing $P^{32}$, in the following manner. Cells were grown in the usual media at 30° C. in the presence of $P^{32}$ (activity, $5 \times 10^5$ cts./min./mg. P). This resulted in complete equilibration of the labeled phosphorus in all fractions. After 48 hours these cells were harvested, washed three times in unlabeled M/15 $KH_2PO_4$, resuspended in unlabeled M/15 $KH_2PO_4$ with 4% glucose, and allowed to ferment the carbohydrate under completely anaerobic conditions. No budding or increase in protein nitrogen is observed in such suspensions. Samples were withdrawn at intervals for activity measurements. It was found that within four hours about one-half of the total P content of the cells. The total activity was found that (except for 1 or 2%) this loss in activity could be completely accounted for in the acid-soluble fraction which forms about 50% of the total P content of the cells. The total activity ($P^{32}$ content) as well as total $P^{31}$ of the nucleoprotein fraction had actually increased slightly (8%) during this period, indicating flow of phosphate into this fraction. These data clearly showed that rapidly metabolizing but nondividing cells did not lose phosphate from the nucleoprotein (NP) fraction even though the major portion of the remaining phosphate was being rapidly equilibrated. Since activity of the phosphate in the acid-soluble fraction of such cells was about one-fourth that of the NP phosphate, they were favorable material for the further study of exchanges between the two fractions. Allowing such cells to ferment carbohydrate for longer periods of time (up to six hours) again left the total activity of the NP fraction unchanged, although the specific activity was decreased slightly owing to dilution by the flow of low-specific-activity phosphate from the acid-soluble fraction.

The entire behavior of the NP fraction was changed, however, when such cells were induced to form new protein either by adding ammonia or by forcing the synthesis of a new enzyme. The results obtained in a typical experiment are exemplified by

the data in Fig. 8. In this experiment cells were suspended in physiological saline containing: (a) glucose, (b) glucose + $(NH_4)_2SO_4$, (c) glucose + $(NH_4)_2SO_4$ + $NaN_3$, (d) glucose + $(NH_4)_2SO_4$ + dinitrophenol. The amount of $(NH_4)_2SO_4$ was equivalent in nitrogen to 50% of the nitrogen content of the yeast. The concentrations of $NaN_3$ and dinitrophenol were $5 \times 10^{-3}$ and $5 \times 10^{-4}$ respectively, sufficient to completely inhibit enzyme formation.

It will be noted that with glucose alone there was no change in activity, whereas when ammonia was present, with consequent budding, the nucleoprotein P dropped to 38% of its original total activity,
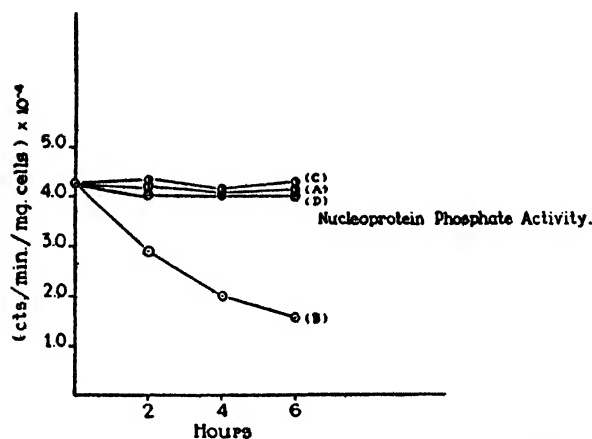


FIG. 8. Flow of nucleoprotein phosphate in cells suspended in (A) glucose, (B) glucose + $(NH_4)_2SO_4$, (C) glucose + $(NH_4)SO_4$ + $NaN_3$, (D) glucose + $(NH_4)SO_4$ + dinitrophenol.

indicating a flow of phosphate from this fraction. It is evident that the azide and, to only a slightly lesser extent, the dinitrophenol were able to prevent this utilization. Except for the fact that the transfer of less phosphate was involved, the same phenomenon was observed when cells were induced to form a new enzyme. Thus, in an experiment in which cells were adapted to maltose, a 34% drop in activity of the nucleoprotein phosphate was observed. Again azide and dinitrophenol in the above concentrations prevented both the formation of the enzyme and the transfer of phosphate from the nucleoprotein fraction.

These findings provide us with the following correlations between protein or enzyme syntheses and the transfer of phosphate from the nucleoprotein fraction:

(1) Rapidly metabolizing cells, which are not synthesizing new protein, do not transfer phosphate from the NP fraction.

(2) Synthesis of new protein or enzyme is always paralleled by a marked transfer of phosphate from the NP fraction.

(3) Agents that are effective in inhibiting enzyme formation and protein synthesis also prevent flow of P from the NP fraction.

To these must be added the fundamental observations of Caspersson and his collaborators on yeast (2), as well as on many other cells, which point to a rigid connection between nucleic-acid metabolism and protein synthesis.

## A PROPOSED FUNCTION OF NUCLEOPROTEIN IN ENZYME SYNTHESIS

The data presented in the previous section leave little doubt that nucleoprotein metabolism is essential for the synthesis of enzymes and proteins. The question that naturally arises is what role the nucleoprotein plays in these synthetic processes. Is it a matter of energy supply, or substrate, or specificity?

No definite answer is available. However, modern biochemical research (13), which has emphasized the role of organic phosphate bonds as sources of energy for synthetic activities, provides a foundation upon which may be based a reasonable hypothesis of nucleoprotein function in enzyme formation. Of particular value here is the mechanism of complex polysaccharide synthesis, the elucidation of which we owe to the brilliant work of the Coris (3) and their collaborators. Two things are required in the formation of a compound like glycogen. One is, of course, the basic hexose unit. The other is the energy necessary to form the bonds linking these units into the complex polysaccharide. The fundamental contribution of the Coris was to show that in the synthesis of a glycosidic bond, glucose-1-phosphate rather than glucose is the reactant involved. This phosphate ester of glucose (Cori ester) already contains in the (C-O-P) link the amount of energy required for the formation of the glycosidic bond. This makes it unnecessary to involve some other compound as an energy donor for the purposes of driving the reaction towards synthesis. The unique feature here is the conversion of an energy-requiring synthetic step into a spontaneous reaction by supplying the necessary energy in the *molecular structure of one of the reactants.*

There seems little doubt that this finding can profitably be accepted as a model on which may be based efforts towards the elucidation of other complex biological syntheses. Several important consequences immediately flow from the adoption of this point of view. It appears that the quantitative energy requirement for a particular synthetic reaction is not the crucial issue in determining its mechanism. There are many phosphorylated compounds (*e.g.*, di-P-glycerate, adenosinetriphosphate) which have more than sufficient energy to form a glycosidic link if there actually existed some mechanism for "feeding" it directly into the reaction. No such mechanism exists, however, and

therefore the energy content of these compounds cannot be used for this purpose. Clearly, in addition to the purely quantitative aspect, there is what may be called the "specificity" of the bond energy. Thus, the actual nature of the bond, and the compound carrying the energy, will determine its suitability for driving a particular reaction. The energy generated by the "catabolic wheel" and trapped in such energy accumulators as adenosine-triphosphate or creatine-phosphate cannot be used as such in driving all the various synthetic mechanisms of anabolism. The energy contained in such compounds must first be transferred to others, which can then act as specific energy donors for particular synthetic reactions.

This concept unifies and simplifies the problem of biological synthesis, since it avoids separating the problem of synthesis into one involving the reactants and another concerned with the source of the "coupled" driving energy. This principle tells us that the solution of one necessarily leads us to the solution of the other aspect of the problem, since they are one and the same.

From this point of view and the established importance of phosphorylated compounds in synthetic reactions, it is not surprising to find that nucleoproteins are controlling elements in enzyme synthesis. We may further plausibly suggest that these phosphorus-containing proteins are the specific energy donors which make possible reactions leading to protein and enzyme synthesis.

### EFFECTS OF NUCLEOPROTEIN FRACTION EXTRACTS ON ADAPTATION

One must be cautious in drawing any final conclusions about the precise role of nucleoprotein either from the preceding data or from the discussion. Even granting that nucleoproteins are directly involved in the synthesis of proteins, it does not follow that they necessarily intervene in the final steps leading to the conversion of such protein molecules into enzymes.

Clearly, some demonstration of *specific* influence by the nucleoprotein on enzymatic constitution would be necessary, before one could draw conclusive inferences of direct determination of enzymatic specificity by a nucleoprotein component. The most critical experiment one could offer in this direction would be one analogous to the already classical investigations of Avery and McCarty on pneumococcus transformation; i.e., the induction of a particular enzyme with a nucleoprotein component in a cell lacking the homologous gene.

No such experiments have yet been successfully concluded with yeast. However, some results of a very preliminary nature have been obtained recently, which bear on this question and warrant mention here. It was reasoned that, if the nucleoprotein fraction (NP) was specifically concerned with enzyme synthesis, it should be possible to observe specific effects of such fractions on adapting cells. Various types of fractionation were tried, and I shall here report briefly on the procedure that yielded the most active preparations.

Cells were extracted with $NaHCO_3$ at pH 9.0 for 3 hours at 30° C. with constant stirring. On removal of the cells the supernatant was adjusted to pH 3.5 and the resulting precipitate washed and redissolved. This was followed by two subsequent precipitations.

The activity of these fractions was tested in the following manner. Unadapted cells were suspended in buffer at pH 7.8, to which had been added the adapting substrate, biotin and pantothenic acid, 5γ/cc., and 1 mg. of nitrogen per cc. in the form of $(NH_4)_2SO_4$. The last three compounds were added to insure that a source of nitrogen was available and utilizable. It was experimentally found that the presence of these substances provided the optimal conditions for testing activity, since certain preparations which were inactive in their absence were extremely active when they were added.

It was found that, when cells adapting to galactose were incubated with such NP fractions prepared from galactose-adapted cells, the adaptation time could be cut down from 180 minutes to 20 minutes. Similar preparations from normal unadapted or maltose-adapted cells possessed no stimulatory activity towards galactose-adapting suspensions. In a similar manner fractions from maltose-adapted cells were only capable of stimulating adaptation to maltose, and the formation of this enzyme was not affected by the addition of extracts from galactose-adapted or normal cells.

That the enzyme itself is being transferred to the unadapted cells in this fraction seems very unlikely from several experiments. The fractions possessed no enzymatic activity by direct test. No maltase activity could be detected in the NP fraction from maltose-adapted cells, nor could the similar fraction coming from galactose-adapted cells influence added galactose. More decisive, however, is the fact that neither could confer the specific activity on active glucozymase extracts obtained from unadapted cells. Neither, therefore, contained in detectable amounts an apoenzymatic or coenzymatic component of the specific enzyme involved.

The conditions for obtaining uniformly active preparations have not yet been completely determined. Since the discovery of this phenomenon about 5 months ago, 32 separate preparations have been made. Of these, 21 have exhibited specific activity in increasing rate of enzyme synthesis. The range of activities observed in the active preparations has been large, extending from 50γ to 0.001γ per cc. for obtaining maximal stimulation. Nothing is known at present about the actual active component in this mixture.

In view of our ignorance of the nature of the

active component, it is clear that nothing can be said about identifying it with nucleoprotein. The interesting and suggestive fact to emerge from these experiments is that it is possible to extract in the nucleoprotein portion a component peculiar to adapted cells, which can specifically stimulate the formation of the same enzyme contained in the cells from which the fraction originated. Until further information is obtained on its biochemical identity we may call this active component by the neutral term "adaptin."

## A Theory of Gene Action

As was pointed out in the introduction, the primary purpose of the present investigation is to acquire information that may lead to an adequate concept of gene action. It is proposed in the present section to suggest a mechanism of gene action based on the experimental findings described here, as well as on previously available information. It need hardly be emphasized that the mechanism to be described must be regarded only as a tentative working hypothesis—its usefulness to be assessed in terms of its success in unifying the diverse data on hereditary phenomena and its fruitfulness as a guide to future experiments.

We may summarize the important findings and conclusions relating to genes and enzyme formation by the following statements.

(1) Under normal conditions the transmission of characters (enzymes and the products of their activities) follows the classical Mendelian laws derived from the assumption that the controlling units are self-duplicating entities, genes, located on chromosomes in the nucleus.

(2) The existence of a particular gene in the nucleus of a cell does not guarantee that the corresponding enzyme will be found in the cytoplasm, as evidenced by such phenomena as cellular differentiation and enzymatic adaptation. Genes, therefore, have as their primary function the indefinite retention for the cell of the *potentiality* for enzyme formation. Certain recessive genes (*e.g.*, the melibiase locus in *S. cerevisiae*) do not possess the capacity for effectively performing this function.

(3) The actual formation of an enzyme in the cytoplasm is mediated directly by a cytoplasmic unit (plasmagene) which possesses the capacity for self-duplication in the presence or absence of the corresponding gene. (Following Wright, *40, 41*, the term "plasmagene" is adopted for the cytoplasmic self-duplicating entity postulated here.)

(4) The presence of the homologous substrates greatly accentuates the capacity of these self-duplicating plasmagenes to produce enzyme.

(5) Competitive interactions exist among the cytoplasmic enzyme-forming units.

(6) Nucleoproteins are involved in the synthesis of enzymes.

Obviously, the view adopted concerning the

identity of the cytoplasmic self-duplicating unit will in large part determine the nature of the hypothesis devised to explain the mechanism of genic control of enzymatic constitution—particularly since the same hypothesis must afford some understanding of the relations between such units and the genes.

It would be hazardous at present to attempt to offer a definitive formulation of what we mean by a "self-duplicating" unit. However, one attribute such a unit is likely to possess is the ability to transform and accumulate energy within its own molecular structure which can be used for the synthesis of similar units. At any rate, it is relatively easy to show that the growth kinetics of such "energy accumulators" is of the self-duplicating or autocatalytic type. Of all the proteins, then, those which would be most likely to be self-duplicating are those which are involved as energy donors in protein or enzyme synthesis. Presumably these proteins could, in addition to aiding the formation of other proteins, drive protein-synthesizing reactions towards the formation of units like themselves.

In view of our interpretation of the role of nucleoprotein in protein synthesis, and of the above discussion, it seems reasonable provisionally to identify the self-duplicating plasmagenes which mediate enzyme synthesis with the nucleoproteins.

Such a hypothesis would be in harmony with the findings that all accepted self-duplicating entities have been found to be linked with nucleic-acid-containing compounds; among such entities may be mentioned genes, plastogenes, viruses, and the pneumococcus "transforming principle."

It must be emphasized that stating that these cytoplasmic self-duplicating units are nucleoprotein in nature does not imply that all nucleoproteins are capable of self-duplication.

Identifying the cytoplasmic unit with the nucleoprotein, rather than with the enzyme as had been done in earlier publications, has several theoretical and experimental consequences. It would be expected that cells not possessing the initiating gene for a particular enzyme could still retain capacity for synthesis of this enzyme, even in its absence, provided an adequate number of the appropriate nucleoprotein units were present. It will be recalled that experiments with melibiase are consistent with this point of view. In some of the clones lacking the gene, irreversible loss of potentiality for melibiase synthesis was not obtained until about 20 hours subsequent to the disappearance of all measurable enzyme activity. Thus, for a considerable period of time, these cells retained the capacity for the synthesis of this enzyme in the absence of any evidence for its presence in the cytoplasm. The experiments cited with galactozymase are also suggestive of this interpretation, since they demonstrated that cytoplasmic trans-

mission of the capacity to form enzyme can occur in the absence of any measurable enzyme activity.

From a theoretical point of view, the nucleo-protein concept of the plasmagene more or less dictates its relation to the gene. In view of the presumed similarity between the two, it seems almost necessary to conclude that the self-duplicating nucleoprotein in the cytoplasm, which mediates the formation of an enzyme, is derived from the gene. One immediate value of this conclusion resides in the fact that it provides us with an experimentally analyzable and testable entity, which can bridge the gap between the gene in the nucleus and the enzyme in the cytoplasm.

We are thus led to propose the following concept of gene action. Genes *continually* produce at various rates more or less complete replicas of themselves, which enter the cytoplasm. These replicas or plasmagenes are nucleoprotein in nature and possess
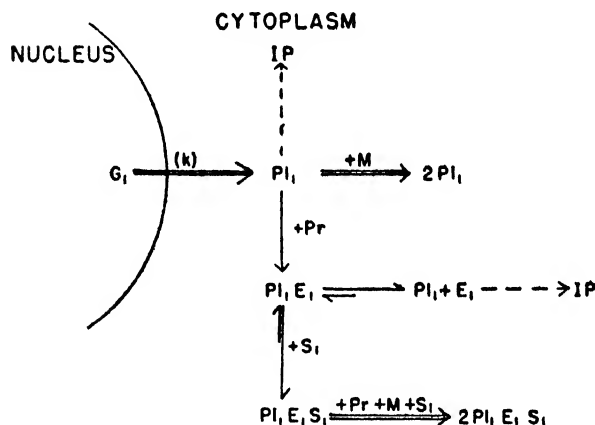


FIG. 9. A mechanism for gene control of enzyme synthesis. The symbols are $G_1$ for gene, $Pl_1$ for plasmagene, Pr for enzyme precursor, $E_1$ for enzyme, $S_1$ for substrate, and M for cytoplasmic material used in duplication of plasmagenes. The constant (k) is a reaction-velocity constant, measuring rate of production of plasmagenes from genes. All double arrows indicate a self-duplicating process; broken-line arrows denote decay to inactive protein (IP) of the elements from which the arrows point. Where forward-and-backward reactions are denoted, the longer of the two arrows indicates the major tendency of the reaction.

to varying degrees the capacity for self-duplication. Their presence in the cytoplasm controls the types and amounts of proteins and enzymes synthesized. These plasmagenes, like all self-duplicating entities, compete with each other for protein and energy, and the outcome of such competitive interactions then determines the enzymatic constitution of the cytoplasm. Inherent in this concept is the possibility of changing the ultimate result of this competition by varying the conditions (*e.g.*, substrates available) under which it takes place. The various reactions and the role of substrate in the process are

detailed in Fig. 9 in terms of one gene $G_1$ and its corresponding enzyme $E_1$.

All the double arrows denote self-duplicating reactions. Gene $G_1$ continually produces its plasmagenes ($Pl_1$) at a rate denoted by k. The plasmagene by its very nature must possess heterocatalytic potentialities; i.e., in addition to being auto-synthetic it must possess the capacity for catalyzing the synthesis of units (enzymes) other than itself. Consequently, once it is in the cytoplasm several things may happen to plasmagene ($Pl_1$). If it is successful in obtaining the proper material (M) in the cytoplasm it will duplicate itself. It may, on the other hand, combine with precursor protein (Pr) and convert it to $E_1$, resulting in the formation of the $Pl_1E_1$ complex.

Since little enzyme is found experimentally in the absence of substrate, one must assume that this complex is highly unstable and quickly breaks up into its two components. A plasmagene once formed cannot, of course, exist indefinitely, particularly in a population of other such units actively competing for the material of which it is composed. The re-action leading from $Pl_1$ to IP (inactive protein) in the figure describes this fact. It will be noted that in accord with the fact described in previous sections the same is assumed to be true for the $E_1$ formed. By IP or inactive protein we mean merely that the plasmagene has broken down to a protein unit which has lost the capacities for self-duplication and enzyme formation, and, in the case of enzyme, into a protein which no longer possesses enzyme activity.

Thus far we have described the reactions that take place in the absence of substrate. It is clear that little enzyme would be found in the cell, unless the rate of $Pl_1$ production by $G_1$ were extremely high or the stability of the enzyme or enzyme-plasmagene complex very great. Neither condition is apparently satisfied in the cases of the enzymes reported on here. When substrate $S_1$ is added, however, it will combine with $E_1$ and two things may result. It is well known that the addition of substrate to enzyme stabilizes it against inactivation. Hence the presence of substrate would decrease the rate at which free $E_1$ is converted to inactive protein. More critical, however, is the possibility that $S_1$ would combine with $E_1$ while the latter is still united with $Pl_1$, thus resulting in a $Pl_1E_1S_1$ complex. The substrate now not only would stabilize the enzyme but also could stabilize the unstable plasmagene-enzyme ($Pl_1E_1$) combination. Such stabilizations of unstable complexes by the addition of a third component are quite common in organic chemistry.

Inherent in the very definition of a self-duplicating entity is the concept that, should such a unit undergo a modification at any given moment, all subsequent replicas would bear this modification. Thus when $Pl_1$ exists alone it *duplicates* only $Pl_1$,

but when substrate is added and the stable Pl₁E₁S₁ combination results *this* is now duplicated, which is indicated in the diagram by the double arrow. This scheme provides, therefore, a concrete mechanism whereby substrate can modify competitive interactions between plasmagenes. What is essentially accomplished by substrate is the creation of a new self-duplicating unit, which duplicates not only the plasmagene but also the enzyme corresponding to the substrate added. It is clear that this scheme would fit both the genetic and the kinetic data discussed in this paper.

From an experimental point of view this mechanism suggests that the enzymes of a cell should be found associated with the nucleoprotein components, in agreement with the data recently accumulated on the enzymatic constitution of the nucleoprotein-containing cytoplasmic granules. More important, it offers a possible experimental solution to a very irritating dilemma which attends any effort at identifying a particular biologically active molecule from a population of chemically similar ones. This is well illustrated by the present-day situation of the pneumococcus transforming principle. Only a relatively small number (about 1 in 10,000,000) of all the molecules in an active preparation of desoxyribonucleate is actually capable of inducing the specific transformation. The biological test for the presence of the active principle is so much more sensitive than any available chemical tests that positive identification of the chemical identity of the active molecules is at present impossible. Thus, while there is little doubt that desoxyribonucleic acid is an essential component of the principle, the possibility that, for example, a protein component is associated with it has not been excluded.

It is obvious that any further efforts on our part to identify by chemical means the nature of the "adaptin" in the nucleoprotein fraction will very likely encounter precisely the same difficulties and arrive at the same impasse. The above theory suggests, however, that the biological specificity of the "adaptin" may be employed as an analytical tool by forming the PlES complex. Thus the corresponding enzyme and substrate could conceivably be used as fractionating devices in separating a particular plasmagene from a host of others, which, while they might be chemically similar, would not combine with any but their own enzymes and substrates. A further advantage which is suggested is that the various stages in the fractionation and isolation of a particular PlES complex can be followed enzymatically if one works with a well-defined and easily measured enzyme.

From a more general point of view, the unique feature of the above theory of gene action is that, while supplying a link between the gene and the enzyme, it at the same time predicts that cells with identical genomes need not possess identical enzymatic constitutions. Whether a particular char-

acter (enzyme) will be transmitted from one cell generation to another in a Mendelian fashion will thus depend on the relative rates of duplication of the controlling cytoplasmic units as compared with their rate (k of the diagram) of production from the genome. If the latter is quantitatively determining, Mendelian inheritance will be observed. If the former is determining, the Mendelian picture will be obscured to varying degrees depending on the self-duplicating capacity of the plasmagenes. It is also clear how substrate could so intensify cytoplasmic inheritance of a particular enzyme as to completely obscure the segregation of the corresponding gene.

As a tentative working hypothesis, this theory has the advantage of providing a unified point of view from which such diverse and apparently contradictory phenomena as classical Mendelian genetics, cytoplasmic inheritance, cellular differentiation, and enzymatic adaptation may be analyzed. The basic problem of cancer involves explaining the appearance of a sudden *heritable* change in somatic cells, analogous in several ways to enzyme adaptation or cellular differentiation. It is, therefore, not surprising that cancer investigators were one of the first groups of biological workers to support strongly the suggested existence of a cytoplasmic hereditary unit. An entity of this kind, by being self-duplicating, provides them with another level at which a mutation can take place and be subsequently transmitted via the cytoplasm from one cell-generation to the next.

More or less similar views have been proposed by geneticists. Wright (40) in particular emphasized several difficulties in trying to explain either growth or differentiation in terms of the classical Mendelian concept of the gene. Thus, the assumption that every time a new protein molecule is formed during growth the gene on the chromosome must intervene as a kind of model implies that growth must proceed linearly from a relatively minute portion of the cell. The kinetics of cell growth follow an autocatalytic law and so are not consistent with this. Wright therefore suggested that perhaps "duplicates or partial duplicates of genes reach the cytoplasm when the nuclear membrane disappears in mitosis and that these can produce duplicates in turn, and so on, permitting exponential increase." To explain the fact that cytoplasmic inheritance is rarely observed, he assumed that the self-duplicating capacity of these free genic replicas is subject to decay. Those that retain this capacity indefinitely he called "plasmagenes."

Again, in connection with cellular differentiation Wright (41) pointed out that the heritable stability of the differentiated state is more easily understood if we assume the existence of self-duplicating cytoplasmic components (plasmagenes), which can undergo controlled mutations. Stimulated by the fundamental observations of Sonneborn (18), Darlington (4) also postulated the existence of a

cytoplasmic self-duplicating unit, which he called the "plasmagene" and which he assumed controls heredity at the "molecular level."

It may be of importance for reasons of clarity to emphasize certain differences between the plasmagene concept developed here and those employed by Wright and Darlington. The plasmagene as defined in the present paper is a more or less complete gene-replica, which possesses to a varying extent the capacity to self-duplicate. It is not a special or unique cytoplasmic component in the sense that it is outside normal physiological processes. It is an integral part of the enzyme-synthesizing system and is the normal link by means of which genes can effect control over protein formation in the cytoplasm. Whether or not plasmagenes are "molecular" is not pertinent to their definition. It seems probable, however, that they would perform their synthesizing functions on the surfaces of relatively large particles (cytoplasmic granules), which could provide the necessary protein and energy-rich groups.

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## DISCUSSION

ZAMENHOF: The Delaporte method, mentioned by Dr. Spiegelman for the obtaining of his "nucleoprotein fraction," was devised originally to obtain desoxyribose nucleic acid; the first step of this method is to get rid of ribose nucleic acid by extraction with dilute sodium bicarbonate solution; the extract contains ribose nucleic acid but no desoxyribose nucleic acid. After extraction, the metachromatic granules disappear from the cells but the latter retain their full vitality as long as their desoxyribose nucleic acid content has not been touched. Dr. Spiegelman uses in his method just this first extract and therefore the chances are that his extract contains ribose nucleic acid and no desoxyribose nucleic acid. If this proves to be correct, then his active principle will be basically different from the one involved in the transformation of pneumococci.

If his principle proves to be indeed ribose nucleic acid, then it is perhaps worth mentioning that according to the recent work of Brachet (Enzymologia, 1945) there may be two kinds of ribonucleic acid in yeast: one "granular," which is thrown down when centrifuging at 50,000 r.p.m., and one "free," which remains in supernatant. While the "granular" fraction is always quantitatively constant and may be involved in some important unknown functions, the quantity of the free fraction can be easily decreased by growing yeast on phosphate-free medium; it is to this "free" fraction that Brachet ascribes many metabolic functions which may make it similar to Dr. Spiegelman's active principle.

Dr. Spiegelman's substance is probably quite depolymerized, since he incubates it for 3 hours at 30° at pH 9.5. However, one has to be careful with such a statement. In Boivin's recent work on transformation of E. coli, he uses as much as 1 mg./ml. of his desoxyribose nucleic acid obtained at pH 3.5 (and therefore depolymerized). Had this publication appeared before the work of Avery and McCarty, one would think that a large amount of depolymerized substance is necessary to induce transformation; actually, as we know from the Avery and McCarty experiments, the amounts needed are of the order of $10^{-5}$ mg., but the substance needed must be highly polymerized; evidently in Boivin's work some very small amounts escaped depolymerization.

COHEN: Before the genetic implications of Dr. Spiegelman's exciting experiments and theory are discussed, I should like to make a plea for a more temperate use of the term "nucleoprotein." The nucleic acids exist in most organized cell structures. In actual practice, especially in one involving an isoelectric precipitation of intracellular material at pH 3.5, it becomes very difficult to obtain material which does not contain nucleic acid. But fractions of this sort contain, in addition to nucleic acid and protein, phospholipids, sterols, neutral fats, enzymes, several types of antigens, vitamins, carbohydrates, etc. Thus almost every type of substance is found in these fractions. In other words, depending on your point of view, these materials might be called lipoproteins, glycoproteins, enzymes, etc. To call the fraction which Dr. Spiegelman has obtained "nucleoprotein" is to imply a causal relationship between his phenomenon and nucleic acid for which there is no evidence at present. As a matter of fact, the data directly relating nucleic acid to specifically inheritable phenomena are very sparse in general. The reactions described by Dr. Spiegelman stand on their own feet and gain not at all in interest by allusions to substances which may or may not be involved in the curious effects described.

MONOD: Since the inspiring results and interpretations reported by Dr. Spiegelman are based on experiments with yeasts, it may be of interest to see how they agree with what is known about enzymatic adaptation in bacteria.

In the first place it should be pointed out that the interactions in the formation of different specific enzymes reported by Spiegelman agree very well with the data obtained on bacteria. In fact, it has been shown that such interactions are of very wide occurrence among carbohydrate-attacking enzymes, and that they account for the phenomenon of diauxis. The basic facts concerning this phenomenon have been briefly mentioned in Lwoff's paper. From a rather extensive study of the occurrence and mechanism of diauxis, the essential conclusion to be recalled in connection with this discussion seems to be that all the adaptive and constitutive carbohydrate-attacking enzymes in bacteria may depend on a common mechanism of synthesis, or, more precisely, on a common precursor. A similar situation, as shown by Spiegelman, appears to exist with glucozymase, galactozymase, and melibiozymase in yeast. (It should be noted, however, that the mutual-exclusion effect of different specific enzymes seems to be much more pronounced with bacteria than with yeast,

since in the latter it occurs only in the *absence* of an external supply of nitrogen. Furthermore, this is in accord with the fact that an external nitrogen source is required for the adaptation of bacteria but not for the adaptation of yeasts.)

From the results on bacteria and yeasts, the problem naturally arises of the nature and degree of specificity of the common precursor (which I have called preenzyme) and of the mechanism of its substrate-induced transformation into a variety of specific enzymes. The data on bacteria seem to show: (a) that the mutual-exclusion effect expressed in diauxis results from a competitive interaction of the *substrates* for the preenzyme; (b) that the rate of adaptation does not depend on the degree of saturation of the pre-existing enzyme. Consequently, it was concluded that the formation of the enzyme occurs as the result of a *substrate-preenzyme* combination, and does *not* depend on the *substrate-enzyme* combination.

Clearly these conclusions do not agree with Yudkin's "mass action" scheme of enzymatic adaptation, and it is quite noteworthy that Spiegelman, on the basis of entirely different experiments with another material, should also have been led to discard this theory.

The same conclusions may also appear, at first glance, to disagree with Spiegelman's scheme of substrate-induced adaptation, since here, as in Yudkin's scheme, the rate of adaptation may seem to depend on the substrate-enzyme combination. However, the main object of Spiegelman's scheme is to explain gene-controlled adaptation and the role of the plasmagene. It implies no hypothesis as to the nature or specificity of the precursor. I believe that with a little further specification Spiegelman's scheme could be made to agree well with the conclusions mentioned above. One need simply assume that the formation of the PIES complex results from the reaction Pr + Pl + S = PIES.

The rate of formation of new enzyme—that is, the rate of adaptation—would then depend on the product of the concentrations of Pr, Pl, and S, and also in part on the affinity of Pr for S. Such a scheme would agree with all the data available at the present time on enzymatic adaptation in both bacteria and yeast, and it might lead to a number of conclusions which could be experimentally tested. For instance, it can be predicted from it that the synthesis of enzymes depending on a common precursor might be affected by two types of mutation:

(a) Mutations involving a change in a "specific" plasmagene, which would affect a single enzyme of the group.

(b) Mutations involving a change in the structure of the precursor, which might affect simultaneously several or all of the enzymes of the group.

It may be stated here that a preliminary study of a number of bacterial mutations affecting carbohydrate-attacking enzymes appears to confirm that mutations of type (b) may indeed occur. However, no definite conclusions as to the validity of these views can be drawn at present from experiments with bacteria. Further confirmations will have to be sought with a material on which straight genetic tests can be performed.

REINER: As a matter of fact, it is possible to obtain direct evidence for the inhibitory effect of constitutive upon adaptive enzymes, using direct measurements of adaptation rates instead of growth experiments. For example, we have shown this for the case of glucose and galactose. If washed cells are adapted in the presence of galactose alone, the initial rate of adaptation is about twice as high as when glucose is added, measuring this rate of adaptation by the increase of anaerobic fermentation rate on galactose in a given period of time.

In view of this, it seemed likely that there should be competition between galactose and other substrates than glucose. It also seemed likely that the intensity of substrate competition should be strongest for substrates whose configurations are close to that of galactose, and become progressively weaker as less similar substrates are introduced. This possibility was tested by studying the effect of these substrates on the breakdown of the adaptive galactozymase. We found an appreciable decrease in adaptive fermentation rate when adapted cells were shaken aerobically in phosphate buffer for 4 to 8 hours, and a very much higher rate of breakdown when glucose was added during this treatment. According to our suppositions, substrates like alcohol and pyruvate should have an effect intermediate between glucose and buffer alone. It was found that the curves for alcohol and pyruvate coincided with each other, and fell just about halfway between the other two curves, in accordance with the hypothesis.

With regard to the question of rate-limiting reactions raised by Dr. Monod, I should like to direct attention to the theoretical demonstration by Burton, a number of years ago, that the over-all rate of a chain of reactions depends not only on the rate of the slowest reaction, but on the rate of other components of the chain as well. Burton indicated that the effect of a slow reaction on the over-all rate depended on the rates of the adjacent reactions and on the presence of other slow reactions in the chain. In view of this, it is conceivable that the introduction of one or more new (adaptive) reaction steps might change the characteristics of a chain without necessarily introducing a step slower than any of those already present. This point deserves more careful investigation.

We have some evidence that the adaptive steps in the case of galactozymase constitute a rather short branch close to the beginning of the Meyerhof scheme for glucose. It seems to be most economical biologically if an adaptive substrate is switched into existing metabolic channels as early as possible, in-

stead of requiring the adaptive formation of a large set of new enzymes. This appears to be the case with galactose. The experiments will bear a good deal of repetition before we are entirely satisfied with them. As far as they go, however, they indicate that galactose is first phosphorylated, and that the resulting galactose phosphate is acted upon by an isomerase, which converts it to glucose phosphate. There are some indications that even unadapted cells can carry out the phosphorylation, and that the isomerase is the adaptive enzyme. In any event, galactose would seem to enter the Meyerhof scheme after at most two steps.

In connection with Dr. Spiegelman's experiments with adaptin, it may be of interest to mention some experiments performed in my laboratory about a year ago, whose interpretation seemed rather obscure at the time. Suspensions of *S. carlsbergensis* were incubated aerobically with galactose and buffer according to our usual technique for adaptation experiments. Samples were taken at intervals, the cells removed by centrifigation or filtration, and the supernatant liquid added to fresh unadapted cells in the presence of fresh galactose. The time required for adaptation to begin in the fresh cells was determined manometrically in the usual way. It was found that supernatant obtained before 45 minutes of previous incubation had no effect, but that supernatant obtained after 45 minutes shortened the time of adaptation, the effect increasing with the length of the incubation period. Supernatant after 180 minutes cut the time down from the control value of 90 minutes to about 45 minutes.

It was supposed at the time that the supernatant might contain intermediates of galactose fermentation which would act as fermentable substrates for the unadapted cells (*e.g.,* pyruvate). However, an investigation of the compounds which could be detected in cells and supernatant during various phases of the adaptive process made this seem quite unlikely. In the light of Dr. Spiegelman's experiments, it appears possible that our active supernatants contained enough adaptin to produce the effects which were observed.

The question which was raised concerning the demonstration that adaptin itself contains no enzyme may be answered by referring to some unpublished experiments by Spiegelman. It was shown that an apozymase prepared from unadapted cells would not ferment galactose when supplemented with a cozymase preparation (boiled yeast juice) made from adapted cells. On the other hand, an apozymase from adapted cells would ferment galactose with cozymase preparations from unadapted or from adapted cells. It is clear from these results that unadapted cells already contain all the cofactors necessary for galactose fermentation, while they do not contain the enzyme. The addition of adaptin to a Lebedew juice from unadapted cells should therefore result in fermentation if any

enzyme were present in the adaptin. The negative result conclusively demonstrates the absence of enzymatic activity.

SPIEGELMAN: I shall in what follows consider some of the questions raised in the preceding discussions. One can hardly fail to agree wholeheartedly with Dr. Cohen's plea for caution in using chemically defined terms for biological agents of undetermined chemical constitution. In reporting the facts of our investigations here I have spoken of the "nucleoprotein fraction," by which I meant to imply only that the active principle or process can be localized in that fraction. No conclusive chemical identification with nucleoprotein was made. It is in this spirit that such chemically neutral terms as "adaptin" and "plasmagene" were employed for the biologically active agents. No objection can be raised, however, against speculations concerning the biochemical roles and nature of these agents, as long as care is taken to distinguish theory from fact.

Our work on the chemistry of the active fraction is still in a primitive stage, especially when compared with the remarkable advances recently made in Avery's laboratory on the pneumococcus transforming principle. Under the circumstances, therefore, I cannot agree with Dr. Zamenhof that one can be relatively certain that the preparation contains no desoxyribose nucleic acid. There is little doubt that ribose nucleic is present, but it is certainly impossible to say now whether it is either the active principle itself or a component of it.

Dr. Pontecorvo raised the interesting question why more instances of cytoplasmic inheritance are not observed in the higher plants and animals if genes normally function via self-duplicating cytoplasmic units as is postulated in the theory proposed here.

I don't think it is merely a matter of coincidence that as soon as attention was focused on the mechanism of transmission of characters in the lower organisms (protozoa, rusts, yeasts) cytoplasmic components almost immediately began to exhibit their capacity to dominate the nuclear hereditary units. The "distance," from the point of view of cell generation and extent of differentiation, between an individual and its gametes is almost vanishingly small in these lower forms. Thus, for example, a diploid yeast cell goes through only two divisions, one equational and one reductional, to produce its haploid gametes. Here both types of gamete, and not merely the "female type," inherit quite directly the cytoplasm of the individual from which they arose, by a process which probably leaves its plasmagene population relatively undisturbed. If the cytoplasm possessed self-duplicating units, this type of gametogenesis would certainly tend to augment and intensify any capacity possessed by these units to overshadow the transmission of the nuclear heredity-determining units.

Compare this situation with the relatively complicated series of differentiations which accompany either spermatogenesis or oögenesis in the higher organisms. The cytoplasm of the resulting gametes is highly specialized as a result of these processes, and the final plasmagene population is quite different from the initial one. A rigid selection of the proper plasmagenes must have occurred during differentiation to form a cell so uniquely suited to perform its biological function. In addition to this, it must be remembered that the germ line is isolated as such relatively early in the embryogenesis of higher plants and animals. Consequently, any plasmagene that is to disturb the Mendelian mechanism must survive in the cytoplasm for a relatively long period extending from early in embryonic development to sexual maturity. The chances of any but the "proper" (i.e., necessary for the onset of differentiation in the zygote) plasmagenes surviving this long waiting period and then the subsequent selection accompanying differentiation must be relatively small, although not completely impossible as demonstrated by the existence of maternal cytoplasmic effects. Presumably, the survival of the "proper plasmagenes" is encouraged by providing the appropriate environmental conditions and substrates.

It is not difficult to understand why the higher plants and animals would tend to develop mechanisms leading to rather rigid control over the plasmagene populations of their gametes, and hence to suppression of the tendency for such cytoplasmic units to determine the transmission of characters. The adult, gamete-producing individuals in the higher plants and animals are the result of a long, complicated series of differentiation reactions delicately synchronized in space and time. The disturbance of any one of these steps could lead to the death of the organisms. Insuring the uniformity of the starting zygotic cytoplasm by suppressing all unnecessary, and therefore possibly harmful, cyto-

plasmic elements in the gametes would provide an important factor of safety for the developmental process. Obviously, where developmental processes leading to the adult form are either trivial or altogether nonexistent, as in the single-celled forms, control over gamete plasmagene population would confer relatively little selective advantage.

Dr. Stern raised the question of the relation of the gene dosage effect experiments to the results and theory of gene action presented here. Whether or not a gene dosage effect with a particular gene will be observed will depend on whether the rate of production of the plasmagene from the gene, or the rate of plasmagene self-duplication, is determining. If the latter is low, either because of an inherently poor capacity for autosynthesis or because of competitive conditions in the cytoplasm, then increasing the dosage of this gene in the nucleus will increase the number of the corresponding plasmagenes that will be found in the cytoplasm. On the other hand, no dosage effects will be observed with any gene whose plasmagene possesses a self-duplicating capacity high enough to quantitatively overwhelm the production rate of the gene, in so far as determining the number of plasmagenes in the cytoplasm is concerned.

It should be emphasized that the theory developed here is one concerned primarily with the mechanism of gene *action* and not of gene *transmission*. The present theory assumes that the latter is strictly Mendelian and is supplementary to the established rules of transmission. Inherent in the proposed mechanism of gene action is the possibility of explaining why in certain instances the transmission of a *character* may not follow that of the *gene*. We sometimes call such cases instances of non-Mendelian inheritance. Actually, since classical Mendelian theory refers only to nuclear gene transmission, such phenomena do not represent violations of Mendel's laws.

# INDUCED BIOCHEMICAL MUTATIONS
# IN BACTERIA

E. L. TATUM

The relationship of the gene to the biochemistry of fungi has been established with Neurospora (5) and confirmed in Ophiostoma (12). It has been shown that genes in these fungi control biochemical reactions involved in the biosynthesis of cellular constituents essential for the growth of these microorganisms. If the biochemical abilities of unicellular forms such as bacteria are governed by units or entities analogous to genes and, like genes, susceptible to mutation, one might expect mutations to occur spontaneously in bacteria and to result in the acquisition of heritable characteristic growth requirements.

Detailed analyses of the biochemistry and nutrition of bacteria have led Knight (18, 19) and Lwoff (25) to correlate the occurrence of bacteria in more and more complex environments with losses in synthetic capacity, resulting in increased complexity of their nutritive requirements. This relationship suggests either the lack of selection against, or a selection for, naturally occurring mutant forms with decreased synthetic abilities. When considered with the relative stability of the heritable growth-factor requirements of many strains of bacteria, such as the lactic-acid bacteria, it also strongly suggests that genes control the biochemistry of bacteria in a manner similar to that in Neurospora; that bacteria possess heredity-determining units similar to genes, which are likewise capable of mutation; and that selective forces determine the maintenance or suppression of a given mutation in a bacterial population.

It has now been well established that many characters in bacteria which result from or are associated with altered biochemical properties may vary in bacterial populations. The occurrence of cells resistant to agents such as phage (24), NaCl (10), heavy metal salts (29), and antibiotics (8, 23), has been interpreted as resulting from mutation. Changes to more resistant forms take place in individual cells in a population in the absence of the agent, with the mutant or resistant cells surviving the action of a particular substance. In a somewhat different manner, selection probably explains the isolation of a methionine-requiring strain of E. coli by continued cultivation in media containing methionine and sulfanilamide (20). Methionineless strains of E. coli have been obtained by similar treatment by other investigators (Roepke, and Lederberg, personal communications.) A slight growth advantage in favor of a mutant that no longer continued to synthesize its required methionine would result in its selection. Selection for

resistance to certain other agents may in like manner involve the more or less indirect selection of strains with altered biochemical capacities. The association of tryptophane and complex nitrogen requirements with phage resistance (1, 2) has permitted the selection of mutant strains deficient in the synthesis of tryptophane. This undoubtedly represents selection of spontaneously occurring mutant strains. (See Luria, 23.)

One characteristic property of genes in higher organisms is that their mutation rate may be increased by a number of agents, particularly by radiation. Several investigators have attacked the problem of mutation in bacteria from this aspect, applying essentially the Neurospora techniques for the detection of biochemically deficient strains, modified primarily by the use of cultures of single-colony derivation. Cultures are irradiated, plated out in multiple supplemented "complete" medium to permit the survival and growth of deficient cells, and strains arising from single colonies are tested for growth in synthetic "minimal" media. From cultures arising from X-rayed material, Roepke, Libby, and Small (28) and Gray and Tatum (14) isolated strains of E. coli with specific growth-factor deficiencies. Gray and Tatum also isolated biochemical mutant strains of Acetobacter melanogenum. Roepke et al. obtained similar strains from unirradiated cultures following serial transfer in complete medium, and were unable to establish a significant effect of the X-ray treatment. Further investigations by Tatum (33) and by Roepke (27) have, however, established the fact that X-radiation increases the occurrence of deficient cells in a population. The results are also in agreement with the essentially random effect of X-radiation on gene mutations in general, in that a variety of different deficient strains has been obtained. This suggests the biochemical and genetic independence of the different types of deficiency. The independence of the different deficiencies was further confirmed by the production of variant strains with double requirements by reirradiating strains with single deficiencies (33). This process presumably could be carried to any desired length. Several strains of E. coli with triple requirements have been obtained by Lederberg, working in my laboratory. The frequency of appearance of a somewhat different type of biochemical mutation in Moraxella lwoffi is increased by X-ray treatment (7). This character, the spontaneous appearance of which has been studied by Lwoff and Audureau (3), is the ability to utilize succinic acid. Lwoff and Audureau

have described other similar characters in Moraxella which are of independent occurrence (26), as are the biochemical deficiencies discussed above.

The original investigations of Roepke, Libby, and Small and of Gray and Tatum seemed to suggest that a period of incubation following irradiation was necessary before plating for the isolation of biochemical mutant strains (Table 1). Gray and Tatum obtained no such mutants following ultra-

biochemical mutations in E. coli. He found that at higher X-ray dosages the log survival curve fell below the straight-line relationship predicted by the single-hit theory of bacterial killing by irradiation. The treatment of cells following irradiation seemed to be of importance in their survival. It was shown that mutant cells apparently are more fragile than normal cells, since they were lost on storage at 4° C. or on continued incubation in

TABLE 1. COMPARISON OF FIRST REPORTED PRODUCTION OF MUTANT STRAINS OF BACTERIA

| Organism | Treatment | Cultures Tested | Mutant Strains | | Investigators |
|---|---|---|---|---|---|
| | | | Total | Different | |
| E. coli A.T.C. No. 9723 | 40 kv., 20 ma. X-ray; 15–40 min. exposure. Single treatment, immediate plating. | 4220 | 0 | | Roepke, Libby, and Small (28) |
| E. coli A.T.C. No. 9723 | 7 kv., 9 ma. X-ray. 139 hours in successive treatments of 17 hours each. | 1883 | 8 | 3 | Roepke, Libby, and Small (28) |
| E. coli A.T.C. No. 9723 | No irradiation; 25 serial transfers. | 1994 | 6 | 4 | Roepke, Libby, and Small (28) |
| E. coli (K-12) | Ultraviolet; immediate plating. | 1200 | 0 | | Gray and Tatum (14) |
| Acetobacter melanogenum | Ultraviolet; immediate plating. | 900 | 0 | | Gray and Tatum (14) |
| E. coli (K-12) | 160 kv., 25 ma. X-ray; 45 min. exposure, 4 hours incubation before plating. | 800 | 2 | 2 | Gray and Tatum (14) |
| Acetobacter melanogenum | 160 kv., 25 ma. X-ray; 45 min. exposure, 4 hours incubation before plating. | 575 | 4 | 4 | Gray and Tatum (14) |

violet treatment with immediate plating, while Roepke et al. found none after a short exposure to hard X-rays, but appreciable numbers following long exposure (up to 139 hours) to soft X-rays. Gray and Tatum then obtained mutants with hard X-rays followed by a four-hour incubation period before plating. These results might suggest a delayed appearance of the mutant character, analogous to the delay in appearance of virus resistance demonstrated by Demerec (9) following either X-ray or ultraviolet treatment. In view of the equivalent effects of the two treatments in Neurospora, and in virus resistance in E. coli, a delayed appearance of biochemical mutants in E. coli seemed to be indicated. Although this may still be true, it seems equally likely that a selective killing of mutant cells with ultraviolet and with X-ray under certain conditions could have been primarily responsible for the results in those instances in which no mutants were obtained. Hollaender has described the destructive effect of ultraviolet of longer wave length on E. coli (15); and the break in the mutation-dosage curve in Neurospora has been attributed in part to some such lethal physiological action (16), presumably operating in the cytoplasm rather than in the nucleus. Roepke (27) has studied the mutation-survival ratio in respect to

broth cultures following treatment. Mutants were obtained from populations plated in complete medium immediately after irradiation in saline phosphate buffer, but none appeared in other samples of the same populations plated out after 15 hours' to 8 days' storage (Table 2).

A complete comparison of the efficiency of ultraviolet and X-ray treatment in the production of

TABLE 2. EFFECT OF IMMEDIATE AND DELAYED PLATING ON SURVIVAL OF MUTANT CELLS OF E. COLI (FROM ROEPKE, 27)

| Exp. No. | Age of Culture | Survival Ratio | No. of Colonies Tested | | Stable Mutants |
|---|---|---|---|---|---|
| | | | Plated immediately | Plated after storage | |
| 460 | young | $1/1.1\times10^6$ | 1090 | | 14 |
| | young | $1/1.1\times10^6$ | | 911 | 0 |
| 482 | young | $1/1.3\times10^6$ | 329 | | 8 |
| | young | $1/1.3\times10^6$ | | 125 | 0 |
| 472 | old | $1/1.2\times10^6$ | 188 | | 3 |
| | old | $1/1.2\times10^6$ | | 100* | 0 |

* Incubated in A.C. broth for 3 hours before plating.

biochemical mutations in bacteria is not yet possible. The failure of Gray and Tatum to obtain well-defined biochemical mutants in Acetobacter or in *E. coli* may have been due to the use of too great a killing dose, with greater selective killing of mutant cells, or to destructive cytoplasmic effects of the irradiation. A few strains of Acetobacter were obtained, however, which apparently required for normal growth complex mixtures of amino acids (Gray and Tatum, unpublished results). Since X-ray and ultraviolet irradiation are equally effective in producing mutations in higher organisms and in increasing the mutation rate to phage resistance in *E. coli* (9), both agents might be expected to be effective in the production of biochemical mutants

tive cells incubated several hours in complete medium before plating. These results suggest that the spores are essentially haploid, and that no appreciable delay in appearance of biochemical mutations is found with this organism under the conditions used, since mixed colonies would be expected to be phenotypically wild-type. A delayed manifestation or expression of the deficiencies is possible, but it seems fairly definite that no segregation of normal and deficient genes occurs during spore germination and the subsequent vegetative divisions.

Another type of treatment that has recently been shown to induce gene mutations and chromosome aberrations is exposure to the chemical action of

TABLE 3. EFFECT OF DI-β-CHLORETHYLMETHYLAMINE ON E. COLI 58

| Series | Treatment | | Survival Ratio | Cultures Tested | Mutant Strains Obtained | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Stable | | | | Unstable | | Total | |
| | | | | | Total | | Different | | | | | |
| | | | | | No. | % | No. | % | No. | % | No. | % |
| 1 | .05% | 20 min. | 1/1×10⁵ | 2034 | 5 | .25 | 4 | .2 | 1 | .05 | 6 | .3 |
| 2 | .1% | 30 min. | 1/1.5×10⁶ | 1234 | 5 | .4 | 4 | .32 | 13 | 1.05 | 18 | 1.4 |
| 3 | 1.0% | 30 min. | 1/2×10⁷ | 1182 | 10 | .8 | 6 | .50 | 1 | .08 | 11 | .93 |

Requirements of Mutant Strains Produced

| Series 1 | Series 2 | Series 3 |
|---|---|---|
| proline, 2<br>methionine, 1<br>phenylalanine, 1<br>tyrosine, 1 | proline, 1<br>phenylalanine, 1<br>threonine, 1<br>hydrolyzed casein, 2* | proline, 4<br>methionine, 1<br>pyrimidines, 1<br>purines, 1<br>adenine or hypoxanthine, 1<br>indole, 2 |

* Requirements not completely analyzed.

in bacteria. The conditions of treatment during and after irradiation with ultraviolet may be more critical than with X-rays. In any case biochemical mutations apparently may be produced by ultraviolet treatment. Lederberg (22) has isolated, following ultraviolet irradiation, strains of *E. coli* that require for growth arginine, proline, and cystine, respectively. Burkholder and Giles (personal communication) have recently obtained a number of biochemical mutant strains of *B. subtilis* from material treated with ultraviolet light. This work is of importance from several aspects. It establishes the feasibility of the general techniques as applied to an aerobic spore-former, an organism quite different from *E. coli*. Secondly, it may permit a critical evaluation of the effect of ultraviolet light in producing biochemical mutations in bacteria. Approximately as many mutant strains have been obtained from treated spores as from treated vegeta-

the β-chloroethyl-amines and -sulfides, the nitrogen and sulfur mustards (13). The effectiveness of mustard gas in inducing mutations was first shown in Drosophila. Both types of compound have been found to produce gene mutation in Neurospora, leading to the production of biochemical mutants (Horowitz et al., 17; and Tatum, unpublished. For review, see 5). Di-β-chloroethylamine has been found to be as effective as X-radiation in producing biochemical mutations in *E. coli* (Tatum, unpublished). Table 3 summarizes the results, including the relation of dosage and mutation frequency. It should be pointed out that the mutation frequencies have been calculated both from the total number of mutant isolates and from the numbers of definitely different and presumably independent mutations. It seems possible that the total number of mutant isolates would give a truer estimate of the actual mutation frequency. In the absence of an

appreciable selective advantage in favor of mutant strains in the three- or four-hour incubation period prior to plating—which is doubtful in view of Roepke's results, since any selection might be expected to be in the other direction—the wild-type cells should divide at a rate similar to that of mutant cells, and the original ratios of normal to mutant cells would remain constant.

The types of substance required by induced mutant strains of bacteria include most of the substances that have been identified as growth factors

spond to those of naturally occurring organisms, spontaneous mutation followed by selection has quite probably been responsible for the growth-factor requirements of bacteria in nature. Roepke *et al.* (*28*) have provided some evidence for a slight selective advantage of mutant strains of *E. coli*. Monod has found a methionine-deficient mutant strain with a 36% faster growth rate than the nondeficient strain (see Lwoff, *26*).

It seems probable that gene mutations, rather than gross chromosomal aberrations or deletions,

TABLE 4. MUTANT STRAINS OF BACTERIA WITH SINGLE REQUIREMENTS OBTAINED AFTER DIFFERENT TREATMENTS

| Substance Required | Treatment | | | | Key |
|---|---|---|---|---|---|
| | None | X-ray | Ultraviolet | Nitrogen Mustard | |
| pyrimidine | | 2 | | 4 | |
| purine | | 2, 3* | | 4 | |
| threonine | 1 | 3 | | 4 | 1—Roepke *et al.*—*E. coli* (*28*) |
| proline | 3, 5 | 2, 4 | 5 | 4 | 2—Roepke—*E. coli* (*27*) |
| phenylalanine | | 2, 4 | | 4 | 3—Gray and Tatum—*E. coli* |
| methionine | 1 | 1, 4, 5 | | 4 | 3*—*A. melanogenum* (*14*) |
| tryptophane | 1, 2 | | | 4 | 4—Tatum—*E. coli* (*33* and unpublished) |
| arginine | 1 | 2 | 5 | | 5—Lederberg—*E. coli* (*22*) |
| cystine | | 1, 4 | 5 | | |
| thiamin | 5 | 1, 4, 5 | | | |

of strains of microorganisms found in nature. The requirements of mutant strains are summarized in Tables 4 and 5. Strains with similar requirements have appeared as apparently spontaneous mutations, and as the result of a variety of treatments—X-rays, ultraviolet light, and nitrogen mustard—in one or more strains of *E. coli,* and in untreated as well as in ultraviolet-treated *B. subtilis.* The single requirements of most of these mutant strains obtained in a number of ways suggest independent deficiencies in biosynthetic capacities, and therefore the existence of independent genes determining these capacities. That synthetic capacities other than the one in question in a given strain are unimpaired is supported by evidence of the production of certain other growth factors by mutant cultures, as detected by symbiotic growth of two different mutants in unsupplemented liquid medium and by the development of satellite colonies in mixed cultures in agar medium with a single supplement (*22*). These techniques may also be of value in tests for "allelism" in mutant "genes" affecting the same synthesis.

Since agents known to produce gene mutations are effective in the production of biochemically deficient strains of bacteria, and since the results are in general single biochemical deficiencies, the conclusion seems well founded that genes or analogous heritable units determine the synthetic capacities of bacteria, and probably their other biochemical and physiological characteristics as well. Since such mutants occur and since their requirements corre-

result from the different treatments and are responsible for the biochemical deficiencies, since in the cases so far examined mutant strains of *E. coli* have been found to revert with low but apparently characteristic frequencies. (Ryan and Lederberg,

TABLE 5. MUTANT STRAINS OF BACTERIA WITH SINGLE REQUIREMENTS OBTAINED FOLLOWING ONLY ONE TYPE OF TREATMENT

| X-ray | | | Nitrogen mustard |
|---|---|---|---|
| nicotinamide, 1 | pyridoxin, 2 | glycine, 3* | tyrosine, 4 |
| biotin, 2,3 | leucine, 2,4 | lysine, 2 | |
| *p*-aminobenzoic acid, 2 | glutamic acid, 2 | histidine, 2,4 | |
| pantothenic acid, 2 | glutamine, 2 | isoleucine, 4,5 | |

| Key |
|---|
| 1—Roepke *et al.*—*E. coli* (*28*) |
| 2—Roepke—*E. coli* (*27*) |
| 3—Gray and Tatum—*E. coli* |
| 3*—*A. melanogenum* (*14*) |
| 4—Tatum—*E. coli* (*33* and unpublished) |
| 5—Lederberg—*E. coli* (*22*) |

unpublished results.) Reversion of a chromosome aberration with complete restoration of synthetic abilities is less likely than reversion of a single mutant gene, although the distinction between the two types of mutation is perhaps artificial.

The closer biochemical investigation of the requirements of mutant strains of bacteria is of value:

(1) in the characterization of the strains studied—essential since genetic characterization is as yet impossible in bacteria; and (2), as in Neurospora, in the analysis of the normal biochemistry of the organism, which is interfered with as the result of the gene mutation. The results of such analyses have in many instances brought the biochemical analogies closer to the biochemical effects of known gene mutations in Neurospora, in addition to supporting the correlation of the reactions in bacteria with those in higher organisms. The relationships of proline, glutamic acid, and glutamine have been supported by the evidence obtained with mutant strains of bacteria. This is consistent with the view that in E. coli glutamic acid is a precursor of proline (33) and of glutamine (27). This is apparently similar to the relations in higher animals (30), and fungi (5), but ornithine is probably not involved in the interconversion of proline and glutamic acid in E. coli (33). The synthesis of tryptophane through anthranilic acid and indole as established with Neurospora has been substantiated in bacteria by the use of mutant strains of E. coli (27). It is of interest from a comparative aspect that production of indole by a tryptophane-requiring strain of B. typhosum has recently been demonstrated by Fildes (11). This is good evidence of the role of indole in tryptophane synthesis in bacteria. The biochemical relation of serine and glycine as represented in Neurospora by an alternative requirement for one or the other amino acid (Hungate, unpublished) has been supported by the similar requirements of mutant strains of Acetobacter (14) and of E. coli (17) and is in accord with the demonstrated conversion of serine to glycine in mammalian tissue (31). Although the biochemical conversion of phenylalanine to tyrosine has been demonstrated in higher organisms, the evidence from bacteria suggests that the relation of these two amino acids may not be so direct. In E. coli, mutant strains are known with specific requirements for each amino acid (Tatum, 33 and unpublished) as well as for a mixture of both (27). This may indicate the existance of a common precursor rather than the direct conversion of one to the other. A similar situation may hold in regard to the related syntheses of methionine and cystine. Arginineless strains of E. coli analogous to those in Neurospora are known (27) and their specificities substantiate the biosynthetic sequence found in Neurospora. Roepke (27) has also reported an apparently connected requirement for both citrulline and uracil, with reversion occurring only for both requirements at once (personal communication). This double requirement may have resulted from metabolic interactions. The inhibition of the growth of wild-type E. coli (K-12) by valine and by its keto-acid analogue, and the release of the inhibitions by isoleucine but not by keto-isoleucine (Tatum, unpublished), suggests the inhibition of the amination of keto-isoleucine

by valine and its keto-acid analogue. Bonner (6) has substantiated and expanded this relationship with an isoleucineless strain of E. coli, and this principle as applied to Neurospora has led to an explanation of the apparent double requirement of a Neurospora strain (16117) for isoleucine and valine. This seems to be due to a single genetic block in the amination of keto-isoleucine, which block leads secondarily to a requirement for exogenous valine (6, 5). This example emphasizes the danger of generalizations regarding apparent biochemical relationships of substances required by a given mutant strain, even when satisfactory genetic evidence of a single gene differential is available. Each case will have to be examined on its own merits, as with the complex requirement of a mutant strain of E. coli for a mixture of 7 amino acids (27). Apparently unrelated requirements may also be found to be related biochemically. This is illustrated by the replacement of p-aminobenzoic acid by a mixture of thymine, purines, and methionine for a mutant strain of E. coli (21). This replacement is interpreted as indicating the function of p.a.b. in the syntheses of the other active substances, a conclusion similar to that of Shive and Roberts (32) based on the effects of methionine and purines on the sulfanilamide-p.a.b. inhibition ratio in E. coli.

Mutant strains of E. coli deficient in the synthesis of purines and pyrimidines are known. In one strain adenine and guanine are interconvertible, possibly through xanthine and hypoxanthine, which are also active. In another strain only adenine and hypoxanthine are active. A third strain requires pyrimidines; all purines tested have been inactive. (See Table 3.) Roepke (27) has reported X-ray-induced mutant strains requiring thymine, uracil, and purines, respectively, as well as strains with requirements for purines and amino acids and for uracil and citrulline.

In conclusion, it now seems quite well established that nutritionally deficient strains may occur in a number of representative bacteria, and that they are produced in greater numbers following treatment with agents known to produce mutations in other organisms. The heritable deficiencies thus obtained are biochemically analogous to those associated in Neurospora with single gene mutations. It seems probable, therefore, that the simpler microorganisms such as bacteria have genes, or at least some units or entities analogous to genes in function and reproduction. The main attribute lacking in bacteria which would make them ideal material for combined genetic and biochemical investigation is their apparent lack of a sexual phase, the existence of which would permit their examination by classical genetic methods for the segregation of characters as Mendelian units. In any case the bacteria, with their biochemical and physiological versatility, ease of cultivation and study, and

the fact that very large populations can be employed, may prove excellent material for the study of the fundamental problems of biochemistry and genetics—the nature of the gene and of gene mutation, the study of biochemical systems, and finally the biochemical and biophysical bases of gene and enzyme reproduction and activity.

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## DISCUSSION

LINDEGREN: Dr. Tatum said that there was no "apparent" sexual mechanism in bacteria, and therefore was somewhat more cautious than Dr. Dubos, Dr. Lwoff, and Dr. Luria, all of whom deplored the fact that "there is no sexual mechanism in bacteria." I suppose that they are familiar with the sexual mechanisms which Badian, Dienes, and I have described, and have rejected them. I believe that this negative point of view in the face of evidence which I, for one, consider convincing is decidedly incautious. Perhaps repetitious description of the sexual mechanism which I have described for *Micrococcus ochraceus* (Lindegren, Carl C. Nuclear apparatus and sexual mechanism in a micrococcus. Iowa State College Jour. of Sci. 16: 307-318. 1942.) and which Badian described for *Bacillus subtilis* and Dienes for the pleuropneumonia bacterium may not be so effective in counseling caution as the following legend:

Over a hundred years ago an abundantly growing fungus was first given a formal description. Following this description it was recognized to be distributed throughout the world. It grew in enor-

mous quantities on almost every available substrate. When fire swept Tokyo after the earthquake the city was orange colored owing to the growth of the fungus. Sugar-cane refuse in the tropics was always covered by its thick yellow growth, and masses of fungus standing several inches thick often covered decayed cantaloupes when they arrived in northern cities. It was famous as a pest in bakeries, and cultures of it were available in every mycological laboratory. A little more than twenty years ago one of the foremost American mycologists wrote an interesting article describing it and pointing out that no sexual forms had ever been found associated with the abundant asexual mycelium. He stated that it was one of the best examples of evolution from the sexual to the nonsexual phase and that this evolution had become so perfect that the sexual mechanism was no longer necessary to maintain the organism in nature. Shortly thereafter two other investigators found that the fungus possessed an extraordinarily complex life cycle with a standard, typically heterothallic, sexual mechanism. It now seems clear that the abundance of the vegetative or asexual form of the organism, which is so apparent in the tropics, is due to the reservoir of variants supplied by the sexual mechanism. The fungus was named Neurospora.

A few years later one of the scientists who named it described some experiments in which he had produced hybrids of this organism; and his colleagues in Washington, fearing that his intense application to his work had affected his mind, proposed that he be given an easier post in California, where he spent a year recuperating. We all know now, or at least sincerely hope, that research work on the production of hybrids in Neurospora is not the sign of an affected mind; but in justice to those who thought so it must be pointed out that this view is arrived at only in retrospect. I think one may safely venture the guess that wherever one finds in nature an abundant and overwhelming growth on a haplophase microorganism one can anticipate positive results if he undertakes a diligent search for the sexual mechanism that replenishes the variants. For example, if one were to study the red stem rust of wheat in Canada, he would find practically every wheat and oat plant affected during some years. It is well known, however, that the sexual phase of this organism appears on barberry and barberry is very rare in Canada, although if it were not for the incredible genius of deBary we might still be ignorant of the relation between wheat stem rust and pustules on barberry leaves. The sources of the infection and the sexual mechanism that supplies the variants occur hundreds or thousands of miles farther south. The ratio of vegetative to sexual growth in the wheat rust is on the order of many billions to one, and this may well be the case in bacteria.

TATUM: Dr. Lindegren's point is very well taken, in that the lack of evidence of a sexual phase in bacteria cannot prove its absence. Another reason for my relative caution in not categorically deploring the absence of sexual processes in bacteria arises from some experimental results obtained recently in our laboratory by Mr. Lederberg and myself. These experiments were performed with mixed cultures of biochemical mutant strains of *E. coli*, and the results, which are described in the paper given by Mr. Lederberg, bear directly on the question under discussion.

PIRIE: The suggestion that the mutation-promoting action of mustard gas and nitrogen mustard is due to their being alkylating agents gives some relevance to experiments that Bawden and I have made with plant viruses.

Tobacco mosaic virus and tomato bushy stunt virus are inactivated by nitrogen mustard without apparent change in their serological, chemical or physical properties. Dimethylsulfate and methyliodide act in the same way, although somewhat higher concentrations of these agents are needed. The only difference we have noticed is that bushy stunt is no longer crystallizable after treatment with nitrogen mustard, but crystallizes normally after treatment with the other two agents. Because it seemed possible that the labile chlorine atoms in nitrogen mustard were involved, we tried chloroacetylcysteine, in which the chlorine atom is extremely labile, but this failed to inactivate tobacco mosaic virus. (Hele, T. S., and Pirie, N. W., Studies in the sulphur metabolism of the dog. VIII. The metabolism of glutathione compared with that of other cystine derivatives. Biochem. J. 25: 1095-2001. 1931.)

# THE CLASSIFICATION AND NATURAL RELATIONSHIPS OF BACTERIA

C. B. van NIEL

The species of Blackberry
Are very apt to cross,
Leaving the Systematists
Completely at a loss,
And distinction of varieties
Is really quite imposs.!
      The Tea Phytologist, Vol. X + 1, No. 1,
      p. 13, Cambridge, England, 1939.

1. Everyone who has worked with bacteria is more or less familiar with such foreign-sounding names as *Bacillus subtilis, Pseudomonas fluorescens, Phytomonas hyacinthi, Thiobacillus thiooxidans, Streptococcus lactis, Mycobacterium tuberculosum, Escherichia coli, Proteus vulgaris, Clostridium botulinum, Actinomyces lavendulae,* etc. He is aware that they are used to designate, in a concise manner, bacteria which are characterized by a combination of morphological, physiological, and biochemical peculiarities. It is also generally realized, though sometimes rather dimly, that these names have something to do with "classification," and have resulted from attempts to express not only the presence of special characteristics but also mutual or even "natural" relationships of the organisms in question.

In many instances the person using a certain designation does so merely because it appears on the label of a culture, or because, in some bacteriology course, he has learned to associate a name with a given complex of properties. But there are also those who are responsible for first putting names on labels, or for teaching students the relations between names and properties. This latter group consists of persons with rather divergent, sometimes quite opposed viewpoints and aims, which arise from different interests, ideas, and previous training. And these differences so influence the result that there exists today rather a large number of conflicting approaches to the problem of classification and relationships of bacteria.

During the past two decades I have spent much time thinking about this subject, and in the last few years various aspects have become much clarified in my mind. It is, therefore, a pleasure to communicate the gist of these reflections and the conclusions that seem to follow from them.

Diversity of conclusions generally results from differences in the premises on which the arguments are constructed. These are often implied without being expressed, and hence frequently remain unrecognized. Sometimes they arise from overemphasis on single aspects of a complicated situation,

with neglect of other phases. As long as the basis for the different solutions is not clearly understood, a resolution of the ensuing conflict is well-nigh impossible. On the other hand, an endeavor to understand the foundation and motivation of conflicting views, while not necessarily implying an immediately satisfactory solution, is apt to lead to a clarification of the issues involved, and to pave the way for a rational approach.

As far as the problem of bacterial classification is concerned, I believe that it is possible to develop some ideas that can serve as a background against which present-day controversies may be better evaluated. This will be attempted by a somewhat historical approach. The various advances made in the course of time in the study of bacteria have tended to shift the emphasis from one to another aspect of the organisms. A proper appreciation of the present difficulties can best be gained, therefore, by a consideration of the successive changes and their implications.

2. The human mind, after becoming aware of isolated facts and phenomena, has the tendency to group them in such a way that a relationship is established between these units. From its simplest expression, as in children's play, to its highest manifestations, as found in the formulation of a scientific theory, this mental activity is fundamentally an attempt at a more or less orderly arrangement of separate and distinct entities. The motivating principle may be quite subjective and apparently irrational, or it may lay claim to a certain degree of objectivity and be based upon rational considerations. To the extent to which such objectivity is realized, the result may be described as scientific.

However, it does not follow that there is but one single manner of arrangement which will be necessarily "the best," even if developed by rational minds. The relative merits of different possibilities are largely determined by the purpose that is to be served. A collection of books, for example, can be arranged according to the subject matter, as in the duodecimal system—and one may then, as the story goes, have to search for Wheeler's essay on "The dry rot of academic institutions" under the subject of "Mycology"—or they may be grouped according to the language in which or the period during which they were written, or even alphabetically according to authors or titles (and there are libraries where the "Archiv für Entwicklungsme-

chanik" is placed under the letter W, because the full title reads "Wilhelm Roux's Archiv für Entwicklungsmechanik"), with almost equal justification. A decided preference for any one arrangement is generally determined by personal needs or special circumstances. Also, anyone familiar with the perplexities of card-indexing literature references knows that, in all probability, no two persons will use exactly the same categories for a filing system. The individual is apt to work out a more or less satisfactory system of his own, modified from those used by others in accord with considerations of how his particular requirements can best be served.

This prelude to a discussion of the "classification and natural relationships of bacteria" is intentionally constructed so as to warn the reader that here, also, pragmatic influences may play an important part. But, although it will be readily admitted that in the above-mentioned instances a number of different arrangements can be adopted, and each one logically defended, it could nevertheless be contended that the argument does not apply to the problem of the taxonomy of bacteria. We may expect that a scientific classification of a group of organisms is no longer subject to personal preferences or needs, but is instead largely objective. Unquestionably, the bacterial taxonomist is diligently striving towards this goal. On the other hand, it is equally true that so far the accomplishments in this direction are meager, and that much of what has gone under the name of taxonomy, and has pretended to be a contribution to our understanding of "natural relationships" of bacteria is, in reality, no more than a simple device, useful for the construction of a system of classification which permits the ready recognition and identification of a bacterial culture. It is not hereby implied that such systems are inferior to the "natural" systems of the botanist and zoologist, but merely that they serve a different purpose, and are arbitrary. If they could more generally be recognized as such, instead of being regarded as inviolable expressions of natural laws, it would be easier for specialists with divergent interests to reach a mutual understanding.

3. The need for some system of arrangement, or "classification," of bacteria originated when, during the second half of the 19th Century, students of the bacteria had found by experiment that not all of these organisms look alike or behave in the same manner. With the recognition of existing differences between members of this group came the desire to be able to refer to various representatives by more precise and terse designations. And it is understandable that the expedient of referring to the representatives of the plant kingdom by the use of a generic and a specific name, introduced some centuries before by Linnaeus for the same

purpose, was almost automatically adopted by the bacteriologists. Binominal epithets were thus coined for bacteria which had, for some reason or another, attracted the attention of the investigator. Usually the names were devised with a view to emphasizing a special characteristic or set of characters.

All this was done without paying much attention to the significance which, in botany and zoology, had gradually become attached to this nomenclatorial procedure. In the course of time the use of the same generic name for a group of plants and animals had come to signify acceptance of more than a certain external resemblance of its members; in the genus-and-species concept had become incorporated much of what students of the theory of evolution had postulated or discovered concerning phylogeny. Hurst's definition of a species (*12*, p. 271) gives adequate expression to this view:

"A species is a group of individuals of common descent with certain constant characters in common which are represented in the nucleus of each cell by constant and characteristic sets of chromosomes."

Still better did the advances in phylogeny become incorporated in the more elaborate systems of classification in which genera were combined into tribes, these into families, orders, phyla, etc.

Now, the fact that the bacteria also have gradually been assigned to families, orders, and classes does not imply that our understanding of their phylogeny is on approximately the same level as our understanding of the plants and animals, in spite of the close resemblance of the structure of the systems of classification. Bacterial taxonomy is far more similar to Linnaeus's original system of the plants, in which a number of equivalent units was proposed, each composed of plants with the same number of stamens. Both kinds of system are useful if they permit the rapid assignment of a specimen to a restricted group, but neither can lay claim to representing phylogenetic relationships.

4. It was Ferdinand Cohn who, in 1872 (*7*), first clearly pointed out this fundamental difference between a system of classification for bacteria and those that had slowly developed for plants and animals. The latter were now based upon the developmental history of the organisms, which, as had appeared from the contributions of comparative anatomists and embryologists, reflected their phylogenetic history. But bacteria have, as far as could then be ascertained, so little of a developmental history that it could not be used as a means of differentiation, or give an inkling of phylogeny.

Complaining furthermore of the profusion of names used for bacteria that might differ only by insignificant details, or perhaps not differ at all but merely have been observed and named by investigators who wanted to stress different features, Cohn appreciated the great significance which attaches to

a stable and generally accepted nomenclature. This would serve to eliminate duplication or multiplication of names for one and the same organism. Success in this respect, however, is dependent upon the recognition of previously named organisms. Two prerequisites must therefore be fulfilled in order to insure the success of an attempt to achieve the desired stabilization. Firstly, every named species should be adequately described. Secondly, a satisfactory method should be available for establishing the identity or nonidentity of a newly isolated bacterium with previously described species. Cohn's contributions to both these aspects were, for the time in which he worked, masterly; he furnished sufficiently complete descriptions of several species so that the organisms could be recognized by others, using the same general approach, and he supplied a sort of key for the allocation of a bacterium to one of the six genera which he proposed and consolidated into four tribes. The differentiation of these groups was based exclusively upon morphological characters, and it was only the gross, external shape and size of the bacterial cells which was considered useful. Though he admitted the existence of physiological differences, their evaluation in terms of satisfactory criteria to serve as a guide for a more detailed classification could not, in his opinion, be attempted at the time. It seemed to him quite possible that, with improvements in microscopic techniques, physiological differences would be found accompanied by morphologically detectable ones. And where such differences did not exist, might not the situation then be comparable to that encountered in a number of cases, especially in the cultivated plants? Here, certainly, no one would venture to propose different specific names for two specimens of almond tree, one of which yielded exclusively bitter, the other only sweet almonds, no matter how constant this physiological peculiarity appeared to be. Hence Cohn's reluctance in admitting physiological criteria for species differentiation; it was a caution resulting from an admitted lack of pertinent information concerning the significance of such characters that caused this attitude.

On the other hand, Cohn made it abundantly clear that the morphologically characterized tribes and genera did not and could not have the value of similar entities in botanical and zoological literature. They were to be considered strictly as "form genera."

Perhaps it is to be regretted that Cohn did not, at this point, stress the fact that the morphological criteria were of great value for purely determinative purposes, and for these only, without in any way suggesting "natural affinities." It is true that the context leaves no doubt that he was fully aware of this quite restricted significance as well as of its fundamental desirability. The simple expedient of using a limited number of easily ascertainable form-

groups made it easy to assign the proper generic name to a bacterial culture, and thus aided greatly in accomplishing a rapid identification. But from subsequent developments it is evident that this very important determinative aspect has often been confused or even identified with a taxonomic one; that bacteriologists have come to accept almost implicitly that mere similarity in shape is an index of mutual relatedness, so that it has, for example, become common practice to consider some special spherical bacterium as obviously more closely related to other spherical bacteria than to rod-shaped ones. Thus Micrococcus, at first just a convenient "form genus," a pigeon-hole where the student could find all the known spherical bacteria, gradually became an entity which, in the minds of many workers, had the same significance as the genus Felis or Lathyrus among the animals and plants. And so with other groups. This might conceivably have been avoided if, instead of adapting well-established terms, such as tribe, genus, and species, with their phylogenetic connotations, to the determinative needs, Cohn had used a different nomenclature, not carrying the suggestion of "natural relationship." It is necessary to bear in mind that the almost exclusive use of morphology in plant and animal systematics has been so successful in tracing phylogeny because here the morphological characters are intricately linked with the rather elaborate life histories of the organisms. But this does not apply to the bacteria, where, in most cases, the life history of the individual seems to be confined to alternate processes of elongation and transverse fission. Hence the morphological criteria have, on the whole, a far more restricted significance, and do not justify any conclusions about phylogeny.

5. As time went on more and more bacteria were isolated, studied, and described. Meanwhile, the methods for study gradually became considerably augmented. More refined microscopic techniques, including special staining procedures, disclosed, as Cohn had anticipated, morphological differences in groups previously regarded as homogeneous. It was also discovered that a group of morphologically very similar bacteria could still be composed of types differing in several respects. It is only natural that those investigators who first noticed such differences felt compelled to stress them by splitting accepted genera or species into a number of new ones, and by proposing numerous additions.

In respect to morphology it was no longer adequate to describe bacteria as spherical, rod-shaped, or spiral. The formation of special aggregates, such as chains, clumps, tetrads, or packets among the spherical bacteria came to be accepted as important additional criteria for differential and diagnostic purposes. The characterization of rod-shaped bacteria was amplified by an inclusion of the arrangement of organs of locomotion in the case of motile

representatives, and the ability to produce spores. The new staining techniques, revealing differences in the chemical composition of otherwise similar organisms, were used as a basis for further differentiation, and made it necessary to incorporate in the descriptions the behavior of a bacterial culture when subjected to such procedures.

Then, too, it was found that differences exist in the action of various bacteria on a wide variety of chemical compounds. The search for specific microorganisms capable of decomposing certain organic substances or causing the spoilage of food products, or responsible for the production of special chemicals, led to the isolation and study of more or less well-defined physiological groups and to investigations of nutritional physiology. This not only added to the already considerable number of species and genera, but also greatly extended the possibility for refinements in characterization.

And, last but not least, there was the recognition of bacteria as causative agents of plant and animal diseases. The economic importance of, and the public interest in this field were so great that the amount of work devoted to the various problems hereby opened up soon overshadowed that carried out on other aspects. Particularly in this realm the need for rapid and conclusive identification was imperative. Hence we see an extensive development of diagnostic methodology especially adapted to these pathogens. Afterwards the same methods were applied to nonpathogenic bacteria. And, although the result was that such organisms were thereafter characterized in far greater detail, it nevertheless cannot be denied that the descriptions gradually became stereotyped and that certain outstanding features, which might have been used to advantage for descriptive purposes, receded into the background, being supplanted by routinely determined qualities of often doubtful value.

6. At any rate, the classification of Cohn had thus become too primitive to be very useful any longer. A new and more up-to-date system was badly needed. It was supplied in 1897-1900 by Migula (23), in whose "System der Bakterien" most of the newly acquired knowledge was compiled and integrated. In the main, it represented a continuation and elaboration of Cohn's classification. The number of genera was, of course, increased because, as mentioned above, additional morphological and some developmental characters, such as type of flagellation and sporulation, had since come to light, and could be used for the demarcation of morphological groups.

But in one respect a radical departure from Cohn's principles was here initiated; Migula's system divided the bacteria into two orders, one of which, the Thiobacteria, was intended to bring together in one systematic unit those organisms which Winogradsky (48) had treated in 1888 in a mono-

graphic study under the designation "sulfur bacteria."

It certainly was an unusual group. Its members were, on the whole, considerably larger than the more commonly encountered bacteria, and many presented rather striking shapes. There were the filamentous Beggiatoa and Thiothrix species, which, except for the lack of pigmentation, closely resemble the blue-green algae of the family Oscillatoriaceae. Secondly, the group included red or purple, phototactic bacteria, many of which appeared to grow in characteristic aggregates or colonies exhibiting a primitive developmental history. All of these bacteria can be found in places where hydrogen sulfide is present, and their cells are usually stuffed with sulfur globules, perhaps the most conspicuous morphological character.

Furthermore, the physiology of these organisms is unique. Winogradsky (47, 48) had shown that hydrogen sulfide and sulfur here fulfill the same function that organic substances generally perform in living orgnisms—viz., that of energy source. Thus the sulfur bacteria provided the first example of a new kind of physiology; they were the prototype of that group of organisms, later often referred to as chemosynthetic bacteria, which derive energy primarily from the oxidation of inorganic substances.

Admittedly, there seemed to be sufficient reasons for treating sulfur bacteria as a distinctive group. But why were they set up as an "order"? Migula did not make this clear; the order Thiobacteria was established without so much as an explanatory sentence. In trying to reconstruct the reasons and assess its significance one is forced to the conclusion that this unit, comprising bacteria of quite diverse morphology, could have been conceived and justified only on a physiological basis. This, then, provides the first instance of the application of physiological characters for the creation of a large systematic entity.

Elsewhere (44) I have tried to show how, in the course of time, this apparently sound principle has resulted in developments which illustrate as convincingly as any the state of utter confusion in bacterial taxonomy. Here it may suffice to state that the separation of the sulfur bacteria as a special group has obvious and great advantages for determinative purposes, but that this procedure leads to irreconcilable conflicts if one attempts to apply it to the construction of a single system of classification.

7. There is no evidence to suggest that Migula intended to imply a phylogenetic entity for the new order Thiobacteria. In fact, no one occupied with bacterial classification had tried to deal with the problem of natural relationships within the group. Some investigators had, it is true, discussed the probable affinities of the bacteria as a group to

other living organisms, and had severally reached the conclusion that they should be considered as related to the blue-green algae, to the fungi, or to the flagellate protozoa (*8, 10, 25, 46*). But a deliberate attempt at developing a phylogenetic system of the bacteria themselves was not made until 1909, when Orla-Jensen published his epoch-making contribution (*29*).

In contrast to the earlier systems, Orla-Jensen's classification was based principally upon physiological characteristics. This was a logical consequence of the theoretical considerations that formed the foundation for the "phylogenetic tree." Briefly stated, the reasoning was as follows. The first living organisms on the earth must have developed in an environment where there was neither light nor organic matter. The only organisms known to be capable of growth under such conditions being the above-mentioned chemosynthetic bacteria, it was rational to look upon them as the first inhabitants of our globe. By a logical sequence of deductions, mostly concerned with an evaluation of the increased complexity of the nutrient requirements and the natural habitat of various groups of bacteria, the first "natural system" was devised. Morphological attributes were not neglected, however, and Orla-Jensen pointed out that the postulated succession of bacterial types could, in the main, also be derived from a consideration of the complexity in structure. The emphasis placed on physiological characteristics was reflected in a new nomenclature, which had the great merit of being both simple and rational. The various genera were designated by names concisely describing the physiological and morphological properties of the organisms.

In spite of many criticisms launched against Orla-Jensen's sytem, it has exerted a tremendous influence. Much of the opposition was clearly due to the belief that it threatened the stability of the existing nomenclatorial procedure. No doubt there was some justification for this attitude; the introduction of the new generic names would have made most of the earlier and generally accepted ones obsolete synonyms, though maintaining many of the groups for which they had been used—a step which may well have appeared an unnecessary and dangerous precedent. But this argument is perhaps rather backward-looking; while it aims at perpetuating what once seemed adequate, it loses sight of the possibility that by substituting a rational for an arbitrary nomenclature a more enduring adherence might be achieved. In any event, the deciding factor must be how acceptable Orla-Jensen's system of classification appears at the present time. It had much to recommend it; it is difficult to escape the conclusions if the premises were valid. And in 1909 they may well have seemed unshakeable; certainly nobody had advanced a more satisfactory explanation for the gradual development of life on earth, and it is still held by many of our contemporaries.

Nevertheless, this concept entails some serious difficulties, which in recent years have been recognized and have led to a search for alternative hypotheses. The most fundamental objection is that an organism growing in an environment composed of only inorganic compounds must be capable of synthesizing all its cellular constituents from carbon dioxide as the only carbon source. This requires a highly developed enzymatic apparatus, and it is hard to imagine how such an apparatus could have originated by any scientifically comprehensible mechanism in an inorganic world. Those hypotheses that have tried to account for the existence of living organisms on earth by postulating that originally they were transported from some other planet or solar system by one means or another do not appear adequate. In the first place, their proponents have yet to answer the objection that during transit the germs would be exposed to ultraviolet irradiation of such intensity and for such periods of time that it is well-nigh inconceivable that any germ could reach the earth without having been killed. Even if this objection could be overcome, the fundamental problem of the origin of life would not thereby be solved; it would merely be shifted from one part of the universe to another.

Important contributions to this problem have lately been made by Oparin (*28*) and Horowitz (*11*). The former envisages the possibility that life did not originate in an environment devoid of organic matter, but that a multitude of organic substances, resulting from strictly chemical reactions, had accumulated before living organisms made their appearance. One of the attractive attributes of this view is that under such conditions the organic compounds, not being subject to rapid decomposition by microorganisms—a process with which we are now so familiar—may have persisted for considerable periods of time. As a consequence more and more complex molecules or molecular aggregates could arise through chemical interactions, and with the emergence of such complexes a fortuitous combination of circumstances might have yielded systems with the property of self-propagation. Systems of this sort would initially be characterized by a minimum of synthetic ability, and Horowitz has ably developed the thesis that the emergence of synthetic mechanisms can be understood as a slow, step-by-step process induced by the successive disappearance of individual components from the environment.

I feel strongly inclined to adopt this line of reasoning, because I find it easier to imagine that the substances which we now recognize as common cell constituents for all organisms could gradually have arisen by chemical (i.e., chance) transformations in an ever-changing environment over a period of many millions of years than to accept the spontaneous appearance in an inorganic world of living organisms, equipped with a complete set of synthetic mechanisms, as a scientific explanation for

the origin of life. Supplemented by Novikoff's concept of "levels of integration" (27), asserting that with an increase in the complexity of a system new kinds of properties, not predictable from those of the components, may be manifested, these ideas constitute, in my opinion, a rational basis for reflections on the nature of the living world.

Unfortunately, their tentative acceptance implies the rejection of the theoretical basis for Orla-Jensen's phylogenetic system. This poses the question whether it is possible to construct another one, with Oparin's and Horowitz's hypotheses as a starting point. At present the answer must, I fear, be negative. The mere fact that the now existing bacteria differ widely in their synthetic ability does not by any means justify the conclusion that we only need to reverse the sequence postulated by the Danish master in order to achieve the desired end. It is undeniable that many of the differences in the power of synthesis, exhibited by related groups of organisms, must be regarded as resulting from losses. Lwoff (21, 22), especially, has brought together an impressive body of evidence in defense of this viewpoint. While one line of thought thus would lead one to develop a phylogenetic system for the bacteria in which the sequential arrangement represents an increase in synthetic ability, another one suggests no less forcibly that the order of appearance of the respective types may actually have been the reverse. The only sound conclusion that seems permissible at present is that we cannot yet use physiological or biochemical characters as a sound guide for the development of a "natural system" of classification of the bacteria.

Orla-Jensen's emphasis on the value of physiological and biochemical characteristics has had the salutary effect that the reluctance to use them for purposes of classification was gradually overcome. As early as 1914 Winslow (49) denied that there is a fundamental difference between morphological and physiological properties, "since all are at bottom due to chemical differences in germ plasm. . . ." This same attitude was later reiterated by Kluyver and van Niel in the statement: ". . . after all, these physiological differences must be considered as expressions of variations in submicroscopical morphology." (16) But if such characters are of no help in reconstructing phylogeny, why then welcome their acceptance as an aid in taxonomic procedure? Breed struck at the heart of this matter when he declared in 1928: "I cannot understand why some still raise the specter of the ancient controversy over the use of morphological versus physiological characters in bacterial classification. Why should we care whether one or the other type of character is more useful in defining a genus? Or whether we should use one type of character in defining a genus and another in defining a species?" (4) The answer to Breed's challenge should obviously be formulated more or less as follows: "Be-

cause it is evident that only one of these sets of characters truly reflects phylogeny." However, in the vast majority of cases the morphology of a bacterium is of no more use in this respect than is its physiology, and the search for a basis upon which a "natural system" can be constructed must continue.

8. Meanwhile, the "form genera," initiated by Cohn, had proved very helpful for determinative purposes. It was evident that Orla-Jensen's physiological genera could be equally useful. Those investigators who were interested in the biochemical performances of bacteria were prone to stress this by coining generic names in which such aspects were given due prominence; had, in fact, already done so before Orla-Jensen's system was published, as the names Nitrosomonas, Nitrobacter, Azotobacter, Acetobacter, Aerobacter, Thiobacillus, Photobacterium, Granulobacter, and many others testify. Especially Beijerinck (1), with his wide interests in microorganisms of the most diverse types, had used this approach to a considerable extent. Now the suggestion that such biochemical genera had a definite phylogenetic significance led to trouble.

Certainly no one will deny that the existence of a special group where all of the vinegar bacteria, or nitrogen-fixing bacteria, or luminous bacteria can be found together makes the rapid identification of an organism which obviously belongs to one of them extremely convenient and expeditious. And the success that accompanied the introduction of genera based upon specific biochemical properties induced specialists in other aspects of bacteriology to apply similar methods to the classification of their pets. Consequently a multitude of genera were proposed, each one stressing some conspicuous character of the bacteria incorporated in it. By the side of Cohn's "form genera" and Beijerinck's and Orla-Jensen's "biochemical genera" there appeared "color genera," "disease genera," "nutritional genera," etc., like Flavobacterium, Rhodococcus, Chromobacterium, Rhodospirillum, Phytomonas, Pneumococcus, Haemophilus, and others.

At first sight it may be difficult to understand why this practice aroused opposition, and resulted in controversies concerning the taxonomic significance of the characters used for the creation of genera. It now seems quite clear that the chief reason was that while the proponents of the different approaches could logically claim expediency—but no more!—for their preferences, they actually tried to rationalize them by making it appear as if the criteria had true phylogenetic value. What Cohn had wisely denied his genera was later claimed for similar entities; and Orla-Jensen's arguments professing phylogenetic importance for physiological genera formed a strong basis for opposition to the validity of genera founded on form, color, pathogenicity, etc.

The enormously increased amount of information concerning the bacteria had gradually made Migula's classification as ineffective as Cohn's had been around the turn of the century. What seemed most needed was a larger number of systematic groups, in order to relieve the congestion in the few genera recognized by Migula. The newly proposed genera would have served this purpose quite adequately, had it not been for the fact that, based as they were on special characteristics of different sorts, their unification into higher taxonomic entities, and their separation from one another, proved well-nigh impossible. A choice had to be made from among the diverse criteria used by various workers, and as a consequence the ensuing fight over "principles in taxonomy" had become unavoidable.

9. By this time very few systematic bacteriologists seemed to be aware any longer of the strictly determinative purposes of the older genera, and many never realized that, by and large, the new ones had no deeper significance. It must, therefore, have seemed feasible to construct for the bacteria a system of classification comparable in all respects to those developed for the higher plants and animals, complete with tribes, families, orders, and classes. At any rate, the urgent need for a new system was apparent, and it was Buchanan's (5) outline which became the basis for the classification later adopted by a committee of the Society of American Bacteriologists (50, 6), and finally for Bergey's Manual of Determinative Bacteriology (3), without question the most elaborate of present-day systems. Buchanan's exhaustive efforts in wielding the great majority of bacterial types into a comprehensive system show clearly the difficulties inherent in this problem, and substantiate the statement made above that one of the most serious obstacles was the characterization of various genera on the basis of very different criteria. A single example may suffice as an illustration.

Migula's order Thiobacteria, renamed Thiobacteriales by Buchanan, had comprised the various genera of the colorless and purple sulfur bacteria described by Winogradsky, all of them conspicuous by the size and shape of their cells and the intracellular sulfur globules. It was useful and justifiable as a physiological-ecological group, though it did not in any way represent an entity with phylogenetic significance. Migula had not known of the small bacteria, capable of oxidizing reduced sulfur compounds, which had since been discovered by Nathansohn (26), Jacobsen (14, 15), Beijerinck (1), and Lieske (20), and collected in a new genus Thiobacillus. But Buchanan was confronted with the problem of whether these bacteria ought to be incorporated with the Thiobacteria. In a physiological sense they obviously were "sulfur bacteria." Yet morphologically they looked so similar to the hydrogen-oxidizing and nitrifying bacteria that their segregation in a different order did not appear logical. Hence they were classified with the latter in the order Eubacteriales. And, since they did not store sulfur droplets in their cells, they could be kept out of the Thiobacteriales by the simple expedient of using these inclusions as the distinguishing criterion for the latter order, which thereby ceased to be a strictly physiological-ecological entity but now became partly defined on vague morphological grounds. Meanwhile it had also happened that Molisch (24), studying purple bacteria that thrive in organic media, described a number of organisms which, by their characteristic pigment complex, bear a close resemblance to the purple sulfur bacteria. This resemblance induced Molisch to propose an order, Rhodobacteria, for both the purple sulfur bacteria (family Thiorhodaceae) and the purple bacteria not containing sulfur globules and requiring organic substances (family Athiorhodaceae). The arguments in favor of a relationship among the purple bacteria were at least as strong as those advocating a relationship of the colorless with the purple sulfur bacteria. The first were based upon the occurrence of a specific pigment system, the other on the significance of the peculiar sulfur metabolism. The reluctance to accept either one as the more important led Buchanan to include in the order Thiobacteriales the colorless and the purple sulfur bacteria, as well as the nonsulfur purple bacteria, with the following diagnosis for the order: "Cells various, typically containing either granules of free sulfur, or bacteriopurpurin, or both, usually growing best in the presence of hydrogen sulphide. The cells are plant-like, not protozoan-like, not producing a pseudoplasmodium or a highly developed encysted resting stage. Spores are rarely or never formed." (5, ix, p. 461) Since, except for the pigment system, the cells of Molisch's family Athiorhodaceae are in no way different from those of the majority of representatives of the Eubacteriales, the exclusion of these colored bacteria from the order seems hard to justify, especially in view of the inclusion of the genus Thiobacillus in the Eubacteriales on the principle of cell morphology. But it may well be asked what other arrangement would have been more satisfactory or acceptable. If a logical answer to this question could be provided, the whole problem of bacterial taxonomy would thereby come closer to a final solution. That this is not yet the case is attested to by the many just criticisms that have been launched against Buchanan's classification, especially in the form in which this was later developed in the successive editions of Bergey's Manual, as well as against several other proposals that have since been made.

Analyzing the situation as presented by the above example, it will be clear that the fundamental difficulty results from the necessity to decide which

of the different types of properties used in the delineation of the groups are the most important for the purpose of combining a number of smaller units into larger ones. Specifically, the groups involved had been segregated on the basis of (1) a special sulfur metabolism, (2) cell morphology, (3) the occurrence of a peculiar pigment system; and the intricate mutual "relationships"—i.e., similarities in certain respects—of these groups are indicated in the following diagram.

reduced sulfur compounds, and comprising the large, colorless sulfur bacteria, the purple sulfur bacteria, as well as the Thiobacillus species, side by side with a group of "purple bacteria" where the sulfur and nonsulfur purple bacteria are collected together, and with an assemblage in which the Thiobacillus species, along with the nonsulfur purple bacteria and many other physiologically different but morphologically similar organisms are incorporated. Such a procedure imposes one im-



The most bewildering aspect is that the cross-relations make any other combination or separation appear just as arbitrary as the one Buchanan finally adopted, because each one of the characters has much to recommend it *as a means of identification.*

Formulated in this manner the statement contains, I believe, the answer to the basic problem that has confronted the sytematic bacteriologists. Having been vexed and confounded by this and other, similarly complex situations for many years, and after, often reluctantly, having realized that any one specific arrangement can be criticized severely and soundly by those who advocate the use of still other criteria, it has come rather as a shock of surprise to find that the solution is utterly simple. In the above paragraph the notation "means of identification" has been used deliberately, and instead of the more alluring term "indication of relationship." The implication is that, for the purpose of identification, the use of these criteria is equivalent, and that it depends upon the information available or readily procurable which one will be the most useful. If this be admitted, then the obvious way out of the dilemma is to split up the group not in one, but in all possible ways; or, vice versa, to recognize not one, but all possible combinations as determinatively helpful entities. Thus we could have a group of "sulfur bacteria," comprising all those organisms characterized by their ability to oxidize

portant restriction, however; the resulting groups cannot be considered as "taxonomic entities," as "families" or "orders" in a phylogenetic sense, but must serve exclusively as aids for the ultimate iden tification of an organism.

It must be clearly appreciated that any special feature can be made the basis for creating a separate group with a larger or smaller number of subgroups. There is, for example, no reason whatever why there should not be, in addition to the above-mentioned three assortments in the sulfur and purple bacteria, yet another one combining only the colorless and purple sulfur bacteria which are characterized by the notable morphological appearance due to the intracellular sulfur inclusions. And it is important to realize that this would be not only a subgroup of the "sulfur bacteria" in the above sense, where they are included on account of their peculiar *physiology,* but also a subgroup of that assemblage of bacteria which can be readily differentiated by some conspicuous *morphological* property.

10. What distinguishes this approach from the one previously employed is that not one character takes precedence over another but that full use can be made of each and every property by which an organism or group of organisms can conveniently be separated from others. The controversial issues have always been concerned with the problem in what

order the several available characters ought to be applied. Both the determinative procedure and the systems of classification have been seriously hampered by inconsistencies in the application of differential criteria. This has been ably argued and documented by Prévot (*32*), who concluded it was necessary to use, for the establishment and the grouping of genera, only morphological characteristics. Fully aware of the limitations of this approach, and recognizing the value of Orla-Jensen's biochemical system of classification, Kluyver and van Niel (*16*) proposed the definition of genera on both a morphological and a biochemical basis, while the demarcation of larger units (tribes, families, etc.) was to be restricted to the use of morphological properties.

No matter how rational this solution may have appeared at the time, its application leads to some very serious difficulties, especially when it comes to a separation of the bacteria from certain of the blue-green algae. And no one will challenge the assertion that such a separation is both desirable and concerned with the delineation of large groups! Here, as Stanier and van Niel (*40*) have pointed out, the primary use of physiological characters is essential. That obstacles might be encountered, which would render the restriction to morphology for the diagnosis of larger units unsatisfactory, was recognized by Kluyver and van Niel, as is shown by the following passage:

"In doing so we are fully aware that this choice may appear arbitrary. The resulting system unintentionally suggests that a morphological evolution has been primary and that in the various stages of morphological development an independent, though sometimes parallel, physiological differentiation has occurred afterwards. Yet it does not seem excluded at all that in special cases the order of events has been the reverse and that in reality parallel morphological evolutions have taken place in two physiologically different groups.

"There is, however, only a limited number of examples in which morphological differentiation in a clearly defined physiological group strongly suggests itself, whereas the instances are numerous that a typical katabolic process is found in groups morphologically so unrelated that affinities on the basis of physiology seem fully incompatible with the evolutionary idea. Moreover, in the former case the range of morphological differentiation extends but over a small number of closely related morphological units, as is *e.g.* clearly shown by the groups of purple bacteria. Therefore in a mainly morphological system such physiologically related groups will remain together whilst, on the other hand, in a mainly physiological system morphologically related groups will be widely dispersed." (*16*, p. 386)

Regardless of the references to "evolution," this passage is a rationalization of the adopted principle; since for the construction of a unified system of classification a consistent use of criteria is necessary, an arbitrary choice had to be made, here as in other cases, and the choice was governed by considerations which can be construed as phylogenetically important, but need not be, any more than those of Cohn, Migula, or Orla-Jensen. The thoughtful criticism of Bruce White (*45*) is, therefore, much to the point, especially the comment on taxonomic methods:

"In setting about a classification, two fundamentally different methods of approach present themselves. The first might be described as 'analytic,' beginning with the major cleavages of the bacterial world and thence working downwards by repeated subdivision to the smaller: such is essentially the principle underlying the so-called 'determinative' systems, which aim at the sharpest definition and at allowing the readiest recognition of any named group, species or variety. The system of Kluyver and v. Niel, though it pulls up short at the definition of spacious genera, is clearly committed to this course. The liability of such a system to grievous error is proportional to the ambition of its major subdivisions.

"The second method might be termed 'constructive.' Here the system must develop from multiple foci of intensive study, gradually widening until they become confluent, when an opinion may be formed as to the rational groupings of larger size. Such a system must be built slowly, but it would be laid on sure foundations and its phylogenetic errors would be those of detail only." (*45*, pp. 146-147)

It is not difficult to agree with these remarks. But when the final conclusion is reached—"The present call is not for newer, more ingenious, more pretentious, systems of classification, but for patient and incisive investigation." (*45*, p. 149)—it is apt to leave some readers with the feeling that something may have been overlooked. This is not necessarily the case. Bruce White may have been as cognizant as any that a system of classification for determinative purposes, better than that represented by Bergey's Manual, was as badly needed as was Migula's "System" in 1900, and may have meant his last sentence to apply principally to a phylogenetic system.

For, let it once again be emphasized: the two are not the same, and the problems of ascertaining the identity of a bacterium are apt to be solved by methods, such as determinative keys, which bear no relation to properties of true phylogenetic value.

11. Up to the present time the determinative aspects of bacteriology have always been incorporated in systems of classification in which a gradually increasing range of characteristics has been used for the delineation of groups. That this was advisable follows from the fact that, as more information was gained concerning the properties of various bacteria, this body of knowledge necessitated finer distinc-

tions. The very large number of rod-shaped bacteria with different properties, in Cohn's system designated as Bacterium species, made it desirable to recognize subgroups, and these were partly established, often as genera, on the basis of such features as flagellation, outcome of the Gram stain, metabolism, color, pathogenicity, habitat, etc. To construct a single system in which the different subgroups can be logically separated obviously requires an effective utilization of all those differential characters. But to accomplish this in such a way as to permit the determination of the subgroup to which a newly isolated organism belongs is extremely hard. Bergey's Manual, designed on this basis, provides many striking examples of the difficulties encountered; it will suffice to review a single one.

In the preface to the fifth edition, the method of using the Manual for determinative purposes is illustrated by describing the stages necessary for identifying *Escherichia coli*, a process which appears quite simple and straightforward. However, if one would attempt the identification of *Aerobacter aerogenes*, which is included in the Manual in the same family Enterobacteriaceae, the problem develops as follows. After having decided upon the proper order, Eubacteriales, and not finding endospore production, one must make a choice from among the first 10 families. The organism not being autotrophic, family I—Nitrobacteriaceae—can be eliminated. But not so family II; the culture was isolated from water, and the bacteria grow well in Koser's citrate medium, which contains only inorganic nitrogen. The obvious choice must, therefore, be the family Rhizobiaceae, with the regrettable result that the culture will be misnamed. If it were to be pointed out that the organism is usually considered a member of the Enterobacteriaceae, the victim would be at a loss to understand why it was included in the group characterized as "usually requiring organic nitrogen and frequently parasites, or live on animal products." Furthermore, if one tries to fit *Aerobacter aerogenes* in the proper family, it is found that the organism, well-known for its property of not producing much acid in sugar media (M.R. negative!), is supposed to "produce much organic acid and often visible gas from sugars"!

This is also a good opportunity for remarking that anyone who has cultivated *Escherichia coli* in synthetic media with ammonium salts as the only nitrogen source would easily be led to the conclusion that even this bacterium should properly be classified in the family Rhizobiaceae on the basis of the diagnosis given for that group!

From these examples it is, of course, obvious that the criteria used for the differentiation of the families in Bergey's Manual have been quite unsatisfactory. An unambiguous identification by means of the key in Kluyver and van Niel's "sys-

tem" (*16*, Table 1, pp. 390-391) would have been easily possible; but this only implies that a better use was there made of the available differential characters. The example chosen is fortunate in this respect, because the genera Escherichia and Aerobacter can be adequately defined on the basis of a combination of morphological and biochemical characters. This is not always true in the case of the genera, or even families, now recognized by the Editorial Board of Bergey's Manual. And the reason is not far to seek; it has, in fact, already been hinted at a number of times. It springs from the desire to acknowledge that there is perhaps as much justification for the acceptance—*as an aid in determinative bacteriology!*—of a special assemblage for bacteria that share the property of being plant pathogens (genera Phytomonas, Xanthomonas, Erwinia), or of producing characteristic yellow or blue-violet pigments (genera Flavobacterium and Chromobacterium, respectively), as for the recognition as a genus of bacteria that can grow in mineral media by oxidizing ammonia, or nitrite, or carbon monoxide.

Several years ago Rahn showed, in an important series of critical essays (*34, 35*), that this practice may have undesirable consequences. These always turn up if the characters of a number of genera partly overlap, because in that event the diagnosis of a genus can never be unequivocally formulated so as to include the desired, and exclude rigorously the unwanted organisms. Since, for example, some of the Cellulomonas species, as well as several bacteria belonging to other genera, are yellow, the genus Flavobacterium cannot be simply characterized as consisting of bacteria with yellow pigments. For, in so doing, the bacteria intended to be classified as Cellulomonas species would be partly encountered in at least one other genus. Furthermore, not all cellulose-decomposing bacteria are to be considered members of the genus Cellulomonas; the cellulose-decomposing actinomycetes, as well as the Cytophaga and Sporocytophaga species, must be kept out. This situation, then, is very reminiscent of that previously discussed in connection with the sulfur and purple bacteria.

Consequently it is easy to understand why a number of systematists have insisted on the use of only a small number of properties, which must be independent and consistently applied so as to avoid overlapping, for the creation of taxonomic units above the rank of species. But this, in turn, results in the necessity for greatly limiting the number of such units. Many properties, either conspicuous or readily ascertainable, and therefore especially desirable as accessories for determinative purposes, must thus be omitted, and it is very likely that this is one of the main reasons for opposition against the rationalized (not natural, or phylogenetic) systems of Kluyver and van Niel, or of Prévot (*16, 32*).

12. The limitations thus imposed are the result of adopting a determinative system in which only one single set of keys is used, the latter being derived strictly from the accepted systematic units. The seven orders now recognized in Bergey's Manual, for example, represent groups of organisms which have been assembled on the basis of a certain complex of common characters (at best), and the key for determining the order to which a bacterium belongs is constructed in accordance with these properties. Similarly the keys to the families, tribes, and genera. This feature of the determinative keys frequently necessitates making a choice from among several available characteristics. It has been pointed out before in connection with the confused state in the order Thiobacteriales that this very fact is apt to make the resulting determinative system far less useful than it might be if all the characters could be employed. This, however, would be possible only if more than one key were available, each one taking into account a different set of criteria. It is clear that, as in the case of the sulfur and purple bacteria, this would result in a variety of combinations. As long as each particular combination has to be identified with some systematic unit, such as a genus or family, this procedure is impossible for the very reason of the overlapping of characters. But it has already been stressed that in most instances those units have no more than a utilitarian, determinative value anyway. If this is granted, there need be no opposition to the suggestion that a far larger number of such determinative entities, even though overlapping, be admitted, with the understanding that none of them needs to be confused with a taxonomic unit.

If the phylogenetic affinities of the various groups of bacteria had been definitely established, it should be possible to use the natural system directly for determinative purposes. But even in that event it is not necessarily the best or most efficient approach, as has been recognized by botanists who have constructed numerous determinative keys based on characters that bear no relation to those of phylogenetic importance. Anyone who has used floras which permit the determination of a plant by means of such features as habitat, size, shape of leaf, color of flowers, structure of stem, time of flowering, type of fruit, etc., is familiar with the practical advantages of being able to select those sets of keys that best suit the circumstances, and will appreciate the opportunity hereby offered for checking the result by a number of different approaches. While in the end one thus arrives at a definite species, and may find it described in detail in its proper setting of genus, tribe, family, etc., in a phylogenetic classification, the stages passed through during the determinative process may have involved groupings whereby one alternative choice yields a family, another a genus, or even directly a species. The larger the number of keys, the greater

is the probability that this will be so; but since it is accompanied by an increased freedom in the choice of differential characters it is in no way objectionable. From a practical standpoint the advantages are obvious, and scientifically I fail to find fault with the occurrence of entities of a composite nature in a given key.

13. Out of these considerations evolves, therefore, the proposal that the systematic bacteriologists give up the attempt at equating the construction of a system of classification with a corresponding set of determinative keys, and instead set themselves the task of developing a large number of keys, each one aimed at the segregation of particular groups on the basis of some especially salient character. If the groups so singled out are not considered as "taxonomic units of equivalent rank," this approach can take full advantage of the knowledge of expert specialists without endangering the efforts of those who persistently and patiently try to contribute towards a "natural" system of classification.

Why, for instance, should we not compose separate keys, differentiating the various bacteria on the one hand primarily by their morphological characters (using shape, size, spore-formation, encystment, conidial reproduction, motility, arrangement of flagella, behavior with respect to the Gram stain, or any other quality), or, on the other hand, by physiological properties (*e.g.*, ecology, habitat, temperature characteristics, color, odor, luminescence), biochemical properties (such as relation to oxygen, nitrogen fixation, special substrates that can be attacked, or metabolic products formed, nutrient requirements), or pathogenic and serological properties? Consider the group of red-colored bacteria, including all the purple bacteria, Serratia species, the red-colored Micrococcus, Mycobacterium, and Actinomyces species, the two red species of Propionibacterium, the one red Acetobacter species, *Cytophaga rubra, Clostridium roseum*, etc. This assemblage of red bacteria is certainly far from homogeneous! It does not in the least represent a "taxonomic entity." But no one will deny that a special key to this group would be very useful, and could expedite the differentiation of the individual components by using well-chosen additional properties of the organisms. Similarly, a key for the determination of all bacteria capable of decomposing cellulose—which would deal with a group comprising various polarly flagellated rods and vibrios, aerobic and anaerobic spore-forming bacteria, Cytophaga and Sporocytophaga species, and some few actinomycetes—could tremendously simplify the determination of any organism isolated as a cellulose decomposer. That *Cytophaga rubra* is represented in both groups, and can probably be determined directly as a distinct species by the use of the first, while the second key will necessitate a

specific differentiation of the Cytophaga-Sporocytophaga group, is not a disadvantage, and is logically inherent in this method of approach. Every striking property could be so used for the segregation of a group that can be further differentiated by one or more keys; and Rahn, who has justifiably objected to genera such as Hydrogenomonas, Phytomonas, and Erwinia, would probably not oppose but rather welcome a key for the specific determination of hydrogen-oxidizing bacteria or of bacterial pathogens, provided this would not be the only key available, and organisms with very similar characteristics, but lacking the ability to oxidize hydrogen or to cause a plant disease, could be determined by the use of other keys.

14. Once completed, such a program would accomplish several desirable objectives. First, all the known bacteria would find a place in one or more keys, and consequently be determinable. This is not possible with the existing systems of classification and determinative procedures, because there are a number of organisms whose affinities to others are at present so poorly understood that they cannot be incorporated into any of the acknowledged taxonomic units. The prevailing practice is to place them in one or more appendices to groups with which some sort of relationship may be claimed, though this usually on very meager grounds, and rather because they cannot properly be fitted anywhere else. The demarcation of the groups to which they are thus appended generally excludes them from the group proper, so that by the use of the accepted means of classification they cannot be found at all.

Secondly, it would become possible to abolish taxonomic entities which, although manifestly and admittedly unsatisfactory, are being perpetuated or established for no other reason than that, in their absence, the organisms composing such groups would become indeterminate. A good example is furnished by the situation arising from the dissolution of the order Thiobacteriales, whose members will, in the forthcoming edition of Bergey's Manual, be incorporated in the orders Eubacteriales and Chlamydobacteriales. This leaves a group of quite conspicuous, though little known, colorless sulfur bacteria, described as Achromatium, Thiovulum, and Macromonas species, without an order in which they might be included; they must, therefore, either be recognized as a new order, or else relegated as an appendix to one of the existing orders with the consequent result that no one would expect to find them there. In the meantime, the order Chlamydobacteriales will have to take care of the filamentous sulfur bacteria of the genera Beggiatoa, Thiothrix, Thioploca, and Thiospirillopsis, where these organisms—composing one of the very few groups whose natural relations are beyond a doubt!—are now to be united with some filamentous bacteria, pre-viously the sole members of that order, of completely unknown relationships. This again because, unless they are recognized as members of an existing order, they cannot be determined at all.

A somewhat similar case concerns the order Caulobacteriales, comprising various bacteria which have in common the property of producing a stalk. Since they may be considered as "differentiated," they cannot properly be placed in the Eubacteriales, and none of the other orders can be so defined as to make their inclusion possible. It is very likely that no one feels particularly happy about this order; on the other hand, its representatives would become indeterminate if the order were abandoned.

I hope that it will by now be evident that the multiple-key system solves such problems in a very simple manner. A key to the "sulfur bacteria," not implying a taxonomic entity, will easily segregate the Beggiatoa group and the Achromatium group; both these groups can also be recognized from a key dealing with all bacterial types with an uncommon morphology. The "stalked bacteria" could be the special subject of another key, and they, too, might be included (as a group, not as an order) in the key for conspicuously shaped organisms.

In the third place, the system of "cross-indexing" the bacteria by means of a multiplicity of characters will, far better than the existing systems, bring out common properties. This will tend to direct attention to the possible identity of organisms which frequently are found together in the keys. Where identity is suspected, but not verifiable on the basis of available information, a few additional experiments will frequently suffice to settle the problem.

15. Hard and laborious as the task of constructing sets of multiple keys may prove, it is anticipated that the general principle will be adopted at some future date; the advantages are too obvious. There is, fortunately, no cause for disagreement between individual workers who might propose alternate approaches, since any suggestion contributing some useful method of subdivision, or of separating special groups by means of certain characters, can be incorporated as an additional instead of frowned upon as a conflicting method of treatment. There looms, however, the problem of nomenclature—concerning which agreement, though desirable, may be far more difficult to reach. How shall the entities that can ultimately be recognized as more or less homogeneous groups be designated?

Many sound and simple arguments can be adduced in favor of relinquishing such orders as Chlamydobacteriales and Caulobacteriales, and it seems probable that this will be readily conceded. The retention of everyday names, like "filamentous bacteria" and "stalked bacteria," in substitution for the currently employed Latin terms provides an immediate solution. This practice can also be applied to many another group, as the previously mentioned

keys for the differentiation of "red-colored bacteria" or "cellulose-decomposing bacteria" indicate. But as the number of correlated characters increases, and the entity thus becomes more precisely defined and circumscribed, a different system of designation becomes necessary, because reference to an organism as "the red-colored, cellulose-decomposing bacterium, which moves without flagella by gliding over a solid substratum, does not form microcysts or fruiting bodies, and can propagate in a mineral medium containing certain specified carbohydrates as the only carbon source at temperatures ranging from 0 to 30° C." is evidently too clumsy. The binominal system of nomenclature is so eminently satisfactory a means of concisely indicating properties that its adoption for bacterial nomenclature is understandable.

What is, perhaps, unfortunate is that it was adopted rather than adapted. For with the use of Latin binominals the corollary of the genus-and-species concept was also involved. Rahn (*34, 35*) has pointed out, along with many others, that this concept is not applicable to bacteria. This is nowhere more evident than in the frequent polemics relative to the justification of species or genera. The utter subjectivity of these concepts as applied to bacteria has never been more succinctly expressed than by Benecke (*2*, pp. 212-213):

"What, then, are species, i.e., the lowest of the taxonomic units? The answer is: that which the investigator who proposes the species wishes to include in accordance with his scientific tact. Obviously it is impossible to answer the question in any other way because Nature herself does not create species, but only individuals with their descendants, so-called 'clones.' And the systematist collects such clones into groups or bundles which he calls 'species.' How big or how little he wants to make his packets—that depends upon his scientific attitude, which may vary for different individuals. He would free himself of this subjective delineation of species only in case he would base his deliberations on yet smaller units; *viz.*, upon these clones themselves. But in that event he would have to culture all bacteria as single-cell cultures, and that under every conceivable set of conditions under which they can possibly live; would have to note all their shapes and properties in dependence of these conditions, and incorporate them in the diagnosis of the respective clone. All clones which under these circumstances fail to reveal differences would have to be united into systematic entities, or 'elementary species.' And then he could proceed to group those entities, now more or less free from arbitrariness, into larger ones according to his precepts. In actual practice this is not possible; this need not be documented further, because such a task would have no end. Hence we must, for the practical purposes of sytematics, i.e., in order to achieve a survey of the different types, choose larger taxonomic units."

It could be contended that in recent years the practice of studying clones has gained much ground and that the distinction between species, for example, on the basis of differences in ability to infect certain types of plants or animals (so-called "host-specificity") in otherwise identical bacteria comes very close to the separation of "elementary species." Nonetheless, the subjectivity and its associated arbitrariness is then merely transferred to the higher taxonomic units. And it is very necessary to realize that the fine distinction, occasionally denied the value of "specific" characteristics by systematists, may be of paramount practical importance to specialists who have to deal with groups of organisms in which such distinctions have been made. The medical bacteriologist, treating his patient by the administration of an antiserum, must know the extent of "type specificity" exhibited by the causative organism; the plant breeder, desirous of testing a certain hybrid for resistance to a bacterial disease, cannot expect to conduct conclusive experiments by using clones of the bacterium to which the species of plant was not susceptible in the first place. The studies on phage resistance, of which a number of reports occur in this volume, provide another striking example of the significance which attaches to the rigorous distinction between clones.

Consider also the industrial bacteriologist who attempts to manufacture vinegar by the use of pure cultures of acetic-acid bacteria, or solvents by the bacterial fermentation of carbohydrates. While he may agree with the specific distinction between *Acetobacter aceti* and *A. rancens*, it is equally possible (and justifiable) that he would prefer another classification on the basis of such properties as the ability to grow in media with high alcohol concentrations, or to produce certain specified concentrations of acetic acid. Or he could point out that *Clostridium butylicum* is too broad a bundle of clones to help him in selecting the most promising cultures for his process.

Discontinuation of the terms species and genus for bacteria, along with the introduction of multiple keys, would eliminate some of the difficulties now encountered, because it would insure a far greater autonomy to specialists in dealing with their own groups and their problems, unencumbered by the exigencies of different groups. There would be no need for the sort of consistency required as the foundation for a single system of classification. Whether the further elaboration of a rational nomenclature along the lines laid down by Orla-Jensen, and further expanded by Kluyver and van Niel, would prove adequate, or whether it might even be preferable to drop the use of Latin names with their taxonomic implications, is a matter for future developments. And, while I am fully in agreement with the opinion that stability in nomenclature is of great importance, I must once more insist that, in the long run, it may turn out to be easier

to gain adherence to a more rational, modernized system than to the current one.

16. The development of a more satisfactory determinative system, divorced from the implications of a direct connection with a phylogenetic classification, can be initiated immediately. Our very limited knowledge of the "natural" relationships of the bacteria will not be a stumbling block for the enterprise. Meanwhile, advances in the understanding of bacterial phylogeny will depend upon following such leads as may be discovered from time to time.

At present this field is virtually uncharted. It is true that there are some small groups which, by the existence of multiple common characters, or by the occurrence of some very outstanding property, so strongly suggest a common origin and natural affinity as to make them universally acceptable as phylogenetic units. As examples of the first kind one could cite the lactic-acid bacteria in the sense of Orla-Jensen (*30, 31*), the propionic-acid bacteria, the fluorescent pseudomonads, the Beggiatoa group, and many of the members of the present family Enterobacteriaceae; the second case is illustrated by the group of purple bacteria that is characterized by the presence of a special pigment system which confers upon the organisms the ability to carry out a unique photosynthetic metabolism. Many other groups of supposedly comparable rank do not, I believe, stand the test of a judicious critical analysis. The present genus Acetobacter, for example, with the morphologically so diverse *A. aceti, A. melanogenum,* and *A. xylinum,* corresponds more to a physiological or ecological than to a phylogenetic group. This is true also for the present genus Azotobacter, as is attested by the definition Starkey was forced to use for this entity when questioned concerning the propriety of including the acid-tolerant nitrogen-fixing bacterium, described as *Azotobacter indicum,* in this genus (*41*).

Not much more extensive is our knowledge of the phylogenetic relations of such small, uniform groups to each other or to other groups of organisms. To believe that mere resemblance in morphology represents a sound basis for revealing phylogenetic trends—as is implicit in the arguments of priority of morphological over physiological characteristics—rests on a misconception, of the fundamental value of such characters when successfully employed for the classification of higher plants and animals, where they are intricately linked with the entire developmental history of the organisms. Hence the recognition of, for example, the polarly flagellated bacteria as a special group, rational and convenient as it is for determinative purposes, should not be interpreted as signifying acceptance of a natural relationship. It may well emerge that the actual affinities of certain members of this line must be sought elsewhere. Such would not be sur-

prising in the case of *Azotobacter chroococcum,* with its highly distinctive encysted stage, a typical developmental feature; but also *Pseudomonas lindneri,* the Xanthomonas and Macromonas species, and *Vibrio (Desulfovibrio) desulfuricans* may turn out to be less closely related to one another or to the fluorescent Pseudomonas species than to a number of other and quite diverse types of organisms.

For only one small group of bacteria does a definite relationship to microorganisms outside the bacterial kingdom appear certain. This is the group of colorless sulfur bacteria, comprising the species Beggiatoa, Thiothrix, Thiospirillopsis, and Thioploca. The morphology of these organisms is so distinctive, and the resemblance to the blue-green algae of the family Oscillatoriaceae so complete, as to constitute practically convincing evidence. These sulfur bacteria can, therefore, best be considered as Oscillatoriaceae which have lost the peculiar pigment system. The relationship between the photosynthetic and colorless forms is comparable to that existing between Euglena and Astasia, between Chlorella and Prototheca, or between the Chlamydomonas and the Polytoma-Polytomella groups. Experimentally no one has yet achieved a transformation of blue-green algae into their colorless counterparts, but this can at present be ascribed to the scarcity of pure cultures of these organisms rather than to any inherent difficulties.

It may be readily imagined, as Stanier and van Niel (*40*) have pointed out, that a number of other bacterial types have been derived in an analogous manner from other groups of the blue-green algae. But in the absence of sufficiently pronounced morphological characteristics and in view of the paucity of developmental features in both groups, such relations are, for the time being, no more than speculative. Here, in particular, experimental investigations starting from the photosynthetic forms might yield valuable information.

Apart from the spore-forming bacteria and the encysting *Azotobacter chroococcum,* which exhibit some sort of primitive developmental history, there are two more groups of bacteria in which the generally recognized life cycle is composed of more than the commonly encountered elongation and transverse fission. They are the actinomycetes and the myxobacteria. The very existence of this more complex developmental history has made it possible to approach the phylogenetic problems by means similar to those used for the higher plants and animals. It is no longer the presence or absence of a single static morphological character in otherwise similar groups, but the occurrence or nonoccurrence of a particular stage in the life cycle, which is here taken as an indication of relationship.

Applied to the actinomycetes, this has led to the recognition of a series which, in order of decreasing complexity of the life cycles, consists of the species

Actinomyces, Micromonospora, and Proactinomyces. The tendency of the Mycobacterium species, particularly the saprophytic ones, to develop under certain environmental conditions in a manner which is almost indistinguishable from Proactinomyces has resulted in an extrapolation of this series so as to imply a phylogenetic relation of Proactinomyces and Mycobacterium. A further extension of this line of reasoning—now, however, no longer connected with the developmental history, but restricted to the combination of more or less fixed morphological characters—makes it possible to include the Corynebacterium species, as well as the propionic and lactic-acid bacteria (*43, 33, 16, 40, 17*). Krassilnikov (*17*) has recently concluded that the Actinomycetales, combined with the non-spore-forming Gram-positive rods and cocci, "must occupy in the classification of microbes the position of an independent taxonomic unit parallel with other independent groups." While the present evidence certainly does not contradict, and in many respects supports this conclusion, it must be borne in mind that, as far as the representatives without conidial and mycelial stages are concerned, it is for the time being a likely reconstruction based upon similarities in gross morphology, staining reactions, etc., but without the sanction of developmental histories. The affinities of this group as a whole to other microorganisms is at present an unsettled problem; arguments that in the past have been used to advocate a relationship with the fungi now appear far from convincing.

In the myxobacteria the situation is somewhat similar. The representatives discovered by Thaxter (*42*) are characterized by life-cycle stages which successively consist of vegetative cells with creeping motility and growth by elongation and division by construction, a swarm stage in which many of the vegetative cells act more or less as a unit, and the ultimate fruiting bodies in which the vegetative cells transform into microcysts, each one of which, upon germination, can give rise to a vegetative cell. The recognition of certain cellulose-decomposing bacteria, known under the name of Sporocytophaga species, as myxobacteria with a life cycle in which the fruiting stage does not include a unified fruiting body but is reduced to the production of isolated microcysts (*18, 19, 13, 37, 38, 39*), marked a significant advance in our understanding of the taxonomy of these organisms. A further important step was taken when Stanier (*37, 38, 39*), studying the creeping motility and swarming of Sporocytophaga and Cytophaga species, conceived of the latter as still more primitive or reduced forms in which even the encysted stage had dropped out.

The outside affinities of the group Myxobacteria, too, are not understood. Stanier's view that the members with specialized fruiting bodies represent the terminal phase of "an evolutionary series which failed to develop further [in the direction from unicellularity to multicellularity] and attain a distinctively multicellular level of organization" (*39*, pp. 182-183) appears well-founded, and would preclude affinities in that direction. The nature of the vegetative cell and its method of locomotion indicate a number of possible groups to which the representatives with less complicated life cycles might be related, more especially the Chroococcales (*40*). This, however, is a mere speculation at the present stage of our knowledge. The recent proposal of Soriano (*36*) to unite the myxobacteria, the spirochaetes, and the Beggiatoa group into an order Flexibacteriales is useful as a determinative aid in that it suggests a separate key for all bacteria that are flexible, or have no rigid cell wall. If, however, it is meant to suggest natural relationships between the groups incorporated in this order, it must be deemed decidedly premature.

The above exposé may have shown that our fragmentary knowledge of bacterial phylogeny is far from sufficient to construct anything like a complete system. Even for a general outline along phylogenetic lines, the available information is entirely inadequate. Much of this is, of course, the result of the paucity of characteristics, especially those of a developmental nature. If the studies of Enderlein (*9*) on complex life cycles in bacteria, now generally disregarded on account of fantastic-sounding claims concerning the significance of "structures" in the bacterial cell of a size well below the resolving power of the microscope, were ever to be repeated with more convincing results, an entirely new approach would thereby be opened up.

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## DISCUSSION

Mellon: The question that I would ask of Dr. van Niel will become more intelligible, I feel, if given orientation by the following experimental background.

In his transformation series keynoted by the corynebacteria and progressing through the actinomyces group, it appears consonant that at least some corynebacteria may be transformed to the streptococcal group and thus extend this series in the opposite direction. Our own variability studies on this point began to take definite form about 1912 to 1914. Until recent years this transformation appeared to be discontinuous, as was evidenced by antigenic distinctness of the dissociants in the several strains where it took place.

However, in recent years we have bridged the gap between these genera, chiefly by dissociating intermediate culture phases so clearly related serologically that a substantial antigenic continuity may be said to exist.

Thus the experimental data clearly show that the conventional group-specific antigenic relation exists between a Group A hemolytic streptococcus (Stoddard strain) and a nonhemolytic diphtheroid dissociated from it. That is to say, the latter strain will agglutinate virtually to full titer of the streptococcus antiserum, leaving undisturbed the homol-

ogous antibodies. And, conversely, reciprocation in the opposite direction is decidedly less evident, which is quite in accord with the antigenic pattern under discussion. Absorption tests were confirmatory.

Furthermore, the nonhemolytic diphtheroid has been observed to revert to the parent Group A streptococcus, as well as to *redissociate* to a stage intermediate between a Group A and a Group C streptococcus, a change which subsequently proceeds to a full-blown Group C, animal type. This rather long study, now about 10 years old, is at present in the hands of the editor of the Journal of Bacteriology.

Incidentally, about 1920 this transformation was shown to be implemented cytologically by large gonidial bodies which, under an appropriate succession of differentiating environments, culminated in a tetrad segmentation. Their germination as tetrads, which quickly broke down to diplo-streptococci, then took place and the strains have remained stable ever since.

With these prefatory remarks as a background, may I ask Dr. van Niel if he would be inclined to regard such transformations as being in accord with his phylogenetic views?

VAN NIEL: From my personal experience with corynebacteria, mycobacteria, and streptococci I am inclined to conclude that in these groups a satisfactory differentiation along strictly morphological lines is extraordinarily difficult to accomplish. As a result, one would certainly expect that

through dissociative phenomena the boundaries will become still more diffuse, so that, for example, a morphologically typical corynebacterium may well emerge as a morphologically typical streptococcus. But in view of what has been said about the value of morphological (versus developmental) characteristics, the morphological similarity *per se* is not an unambiguous criterion for phylogenetic relationship.

It would be important to know in how far the biochemical properties of the cultures are influenced by the gradual or sudden morphological variations. I refer especially to those properties that may be considered characteristic for the genus Corynebacterium on the one hand and Streptococcus on the other. Members of the former genus display either a strictly oxidative metabolism (*e.g.*, the "diphtheroids," as contrasted with *C. diphtheriae*), or they may, as is true for *C. diphtheriae* proper, be potentially capable of inducing a rather typical propionic-acid fermentation of carbohydrates. All species are catalase positive. In contrast, the Streptococcus species show a pronounced tendency towards a fermentative metabolism, and this is typically a lactic-acid fermentation. All are catalase negative.

For the purpose of differentation, these criteria are at least as valuable as the morphological ones, and it would add considerably to our understanding of dissociative phenomena and of natural relations if such biochemical characteristics could be included in the description of the variants.

# APPENDIX

## NOMENCLATURE OF NUTRITIONAL TYPES OF MICROORGANISMS

Many designations have been proposed for the delineation of groups of microorganisms on the basis of nutritional requirements. Various authors have defined such terms in different ways. This is confusing, and leads to misinterpretation when a term is used without reference or redefinition.

It has been felt that an international agreement in regard to terms and definitions is desirable. The following terminology is hereby proposed to characterize nutritional types. The segregation of these types is based on their minimal requirements for growth. Certain terms previously used have been redefined, and new ones introduced.

I. Nomenclature based upon energy sources.
  A. PHOTOTROPHY
    Energy chiefly provided by photochemical reaction.
    1. *Photolithotrophy*
      Growth dependent upon exogenous inorganic H-donors.
    2. *Photoorganotrophy*
      Growth dependent upon exogenous organic H-donors.
  B. CHEMOTROPHY
    Energy provided entirely by dark chemical reaction.
    1. *Chemolithotrophy*
      Growth dependent upon oxidation of exogenous inorganic substances.
    2. *Chemoorganotrophy*
      Growth dependent upon oxidation or fermentation of exogenous organic substances.
  C. PARATROPHY
    Energy apparently provided by the host cell.

  1. *Schizomycetotrophy*
    Growth only in bacterial cells.
  2. *Phytotrophy*
    Growth only in plant cells.
  3. *Zootrophy*
    Growth only in animal cells.
II. Nomenclature based upon ability to synthesize essential metabolites.
  A. AUTOTROPHY
    All essential metabolites are synthesized.
    1. *Autotrophy sensu stricto*
      Ability to reduce oxidized inorganic nutrients.
    2. *Mesotrophy*
      Inability to reduce one or more oxidized inorganic nutrients = need for one or more reduced inorganic nutrients.
  B. HETEROTROPHY
    Not all essential metabolites are synthesized = need for exogenous supply of one or more essential metabolites (growth factors or vitamins).
  C. HYPOTROPHY
    The self-reproducing units (bacteriophages, viruses, genes, and so on) multiply by reorganization of complex structures of the host.

Composite names are used for the concise characterization of a nutritional type with respect to the chief energy source as well as to the capacity for the synthesis of all essential cell constituents. Examples:

Photolithoautotrophic—*Chlorella vulgaris.*
Chemoorganoheterotrophic—*Phycomyces blakesleeanus.*

| | | Autotrophy | | Heterotrophy | Hypotrophy |
|---|---|---|---|---|---|
| | | Autotrophy S.S. | Mesotrophy | | |
| Phototrophy | Photolithotrophy Photoorganotrophy | *Chlorella vulgaris* | | *Rhodopseudomonas palustris* *Rhodospirillum rubrum* | |
| Chemotrophy | Chemolithotrophy Chemoorganotrophy | *Thiobacillus denitrificans* *Pseudomonas fluorescens* | *Escherichia coli* | *Saccharomyces cerevisiae* | |
| Paratrophy | Schizomycetotrophy Phytotrophy Zootrophy | | | | **Bacteriophages?** **Plant viruses?** **Animal viruses?** |

The above proposal developed as the result of discussions during the eleventh Cold Spring Harbor Symposium and was elaborated by A. Lwoff, C. B. van Niel, F. J. Ryan, and E. L. Tatum. It was submitted to O. T. Avery, H. A. Barker, A. C. Braun, F. M. Burnet, R. Dubos, Sir Paul Fildes, N. Fries, C. Fromageot, P. H. H. Gray, A. Imsenecki, B. Issatchenko, H. L. Jensen, G. W. Kidder, A. J. Kluyver, B. C. J. G. Knight, A. G. Lochhead, J. Monod, J. H. Mueller, R. Nilsson, S. Orla-Jensen, W. H. Peterson, A. Pijper, E. G. Pringsheim, L. Provasoli, W. J. Robbins, W. Schopfer, Jan Smit, Santos Soriano, Marjorie Stephenson, A. I. Virtanen, Sergei Winogradsky. Their comments or suggestions will be summarized in a later publication.

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